Universidade de Aveiro Departamento de Biologia
2009

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ESTUDO DA EVOLUÇÃO ESPACIAL E TEMPORAL DA POLUIÇÃO POR TBT NA COSTA PORTUGUESA

SPATIAL AND TEMPORAL EVOLUTION OF TBT POLLUTION IN THE PORTUGUESE COAST

tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Prof. Doutor Carlos Miguel Miguez Barroso, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro

Apoio financeiro do POCTI no âmbito do III Quadro Comunitário de Apoio.

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio.

Ciência.Inovação 2010 Programa Operacional Ciência e Inovação 2010
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

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agradecimentos

Ao longo do tempo, várias foram as pessoas que me ajudaram e sempre incentivaram para que o trabalho fosse concluído. A essas pessoas, que de alguma forma deram o seu contributo para a concretização deste projecto, gostaria de deixar algumas palavras.

Ao meu orientador, Professor Doutor Carlos Miguel Miguez Barroso, gostaria de manifestar o meu reconhecimento pela forma como delineou as linhas deste trabalho e pelos ensinamentos prestados durante a sua realização. Não poderia deixar de reconhecer a confiança que depositou em mim, o seu empenho e a sua disponibilidade bem como o facto de me ter acolhido como membro da sua equipa. Pelo constante incentivo um muito e reconhecido obrigada.

À Professora Doutora Eduarda Pereira, do Departamento de Química da Universidade de Aveiro, gostaria de agradecer o apoio, ensinamentos e as condições disponibilizadas para a realização de análises químicas.

Ao Doutor William Langston e ao Doutor Gary Burt (que infelizmente já não se encontra entre nós) muito agradeço a assistência que me prestaram no Laboratório Marinho de Plymouth, Reino Unido,

Ao Doutor Manuel Sobral do Instituto de Investigação das Pescas e do Mar (IPIMAR – Aveiro) o meu agradecimento por me ter proporcionado a oportunidade de participar nas campanhas de amostragem na plataforma ao largo de Aveiro.

Ao Doutor Miguel Gaspar do Instituto Nacional dos Recursos Biológicos (IPIMAR – Olhão) agradeço a oportunidade de ter participado nas campanhas de amostragem na plataforma ao largo de Lisboa, Setúbal e Faro e por ter disponibilizado as instalações do IPIMAR de Setúbal para a realização do trabalho laboratorial.

À tripulação do Tellina e do Diplodus um especial agradecimento por toda a ajuda prestada aquando das amostragens.

À Professora Maria Helena Moreira, do Departamento de Biologia da Universidade de Aveiro, agradeço a revisão cuidada que realizou aos principais capítulos desta tese.

Aos meus colegas de equipa e amigos, Ana Sousa, Isabel Oliveira, Nelson Ferreira, Sónia Coelho, Susana Galante-Oliveira e Raquel Quintã, pelos momentos partilhados, pelas sugestões, entusiasmo, e interesse que sempre demonstraram. Não poderia deixar de expressar um agradecimento especial pela sua amizade incondicional tanto nos bons como nos maus momentos.

Gostaria ainda de manifestar o meu agradecimento aos colegas do laboratório de Química Analítica, do Departamento de Química da Universidade de Aveiro, pelo apoio e ajuda sempre manifestados.

Aos meus Amigos, em quem tantas vezes pensei ao longo destes anos, que sempre me apoiaram e entusiasmaram, compreendendo sempre a minha indisponibilidade.

À minha família pelo apoio, compreensão e paciência que me têm sempre dedicado.

A todos, Muito Obrigada.

A realização deste trabalho contou com o apoio financeiro das seguintes instituições e programas:

- Projecto de investigação POCI/MAR/61893/2004, financiado pela Fundação para a Ciência e Tecnologia e pelo POCI 2010, co-financiado pelo FEDER;

- Bolsa de Doutoramento (SFRH/BD/12441/2003) atribuída pela Fundação para a Ciência e Tecnologia.

- CESAM (Centro de Estudos do Ambiente e do Mar) e Departamento de Biologia, Universidade de Aveiro.

Palavras-chave *Nassarius reticulatus*, *Imposex*, Poluição Marinha, Tributilestanho, Trifenilestanho, Cobre, Parasitas Trematódes.

Resumo

O presente trabalho teve como principal objectivo estudar a evolução espacial e temporal da poluição por TBT na costa portuguesa, utilizando o gastrópode *Nassarius reticulatus* como espécie indicadora. A avaliação dos níveis de poluição por TBT foi principalmente realizada com base na medição do grau de masculinização das fêmeas de *N. reticulatus* (*imposex*) para vários locais e datas de amostragem, dado que o *imposex* é um biomarcador específico da contaminação por TBT. Para avaliar a real dimensão deste problema em Portugal, foi efectuado o levantamento dos níveis de *imposex* ao longo de toda a costa continental Portuguesa, incluindo os principais sistemas estuarinos e algumas zonas da plataforma continental adjacente até à profundidade de 34 m. Tornou-se evidente que *N. reticulatus* é uma espécie indicadora de elevado valor dado que é abundante e tem uma ampla distribuição na costa continental portuguesa, apresentando, para além disso, uma sensibilidade adequada para descrever os gradientes de poluição existentes em toda a área de estudo.

Outras espécies de nassarídeos foram também amostradas ao longo da costa, nomeadamente, *Nassarius pygmaeus*, *Nassarius nitidus* e *Nassarius incrassatus*. Verificou-se que estas espécies são pouco abundantes, tendo apenas sido possível colher um número suficiente de espécimes de *N. incrassatus* em alguns locais da costa Portuguesa para o estudo de *imposex*. Constatou-se que esta espécie indicadora é menos sensível do que *N. reticulatus* para avaliação dos actuais níveis de poluição por TBT, sendo menos adequada para utilização em programas de monitorização nesta costa.

O levantamento de *imposex* de *N. reticulatus* ao longo da linha de costa em 2006 mostrou que os níveis de poluição por TBT, de uma forma geral, são elevados e representam um risco efectivo para os ecossistemas marinhos, sendo este problema mais grave em locais situados no interior ou na proximidade das zonas portuárias. De facto, em cerca de 95% dos locais amostrados *N. reticulatus* apresentou níveis de *imposex* (VDSI > 0,3) que indicam concentrações ambientais de TBT acima do valor de referência definido pela OSPAR (“Environmental Assessment Criteria”), a partir do qual podem ocorrer impactos negativos para algumas espécies sensíveis no ecossistema.

Foi também realizado o levantamento de *imposex* de *N. reticulatus* em zonas mais profundas da plataforma continental da região de Aveiro, entre 2004 e 2006, bem como das regiões de Lisboa, Setúbal e Faro, em 2006. Verificou-se que os níveis de *imposex* e de contaminação por TBT nos tecidos destes gastrópodes estão correlacionados e decrescem com o afastamento às

zonas portuárias, as quais constituem a principal fonte de contaminação nestas áreas de estudo. Apesar da distância aos portos e da dispersão do TBT nas grandes massas de água existentes nestas zonas, estas encontram-se consideravelmente afectadas pela poluição por TBT. De facto, foram observados valores de *imposex* com VDSI > 0,3 em vários locais de amostragem, bem como níveis de concentração relevantes de TBT nos tecidos.

Estudou-se a evolução dos níveis de *imposex* de *N. reticulatus* ao longo dos últimos anos para avaliar se ocorreu recentemente alguma alteração da poluição por TBT na costa Portuguesa. Os níveis de *imposex* registados em 2006 na linha de costa foram comparados com dados obtidos por outros autores na mesma área de estudo em 2000 e 2003. Os resultados demonstraram que ocorreu uma redução dos níveis de *imposex* entre 2003 e 2006, mas o mesmo não sucedeu entre 2000 e 2003. Registou-se igualmente uma diminuição do *imposex* de *N. reticulatus* na plataforma adjacente a Aveiro entre 2004 e 2006 ou entre 2005 e 2006. A principal causa desta evolução foi, muito provavelmente, a entrada em vigor em Julho de 2003 do regulamento EC/782/2003 que proíbe a aplicação de tintas com TBT (ou outros organoestanhos) em qualquer tipo de embarcação. Contudo, torna-se necessário acompanhar por mais tempo a evolução do quadro actual, para o qual os dados obtidos neste trabalho constituem uma base de referência fundamental. Este aspecto é muito importante dado que entrou em vigor em Setembro de 2008 a Convenção Internacional sobre o Controlo da Utilização de Sistemas Antivegetativos Nocivos em Embarcações, aprovado pela IMO (Organização Marítima Internacional), que impõe uma proibição global da navegação de navios com tintas antivegetativas contendo TBT (ou outros organoestanhos).

Face à proibição da utilização de organoestanhos nas tintas antivegetativas e à sua substituição por outros biocidas, nomeadamente os compostos de cobre, levantou-se a suspeita de que os níveis deste metal nos ecossistemas marinhos podiam aumentar. Para avaliar se realmente tem havido um aumento deste metal no ambiente, fez-se um levantamento do grau de contaminação dos sedimentos por TBT e por cobre ao longo da costa em 2006 e compararam-se estes valores com os registados em amostras de sedimentos conservadas obtidas para os mesmos locais em 2000. Os resultados demonstraram que não existem diferenças significativas nos níveis de TBT e de cobre entre aqueles dois anos. Considerando que a poluição por TBT tem vindo a diminuir na costa Portuguesa, a manutenção dos níveis de contaminação nos sedimentos sugere que estes possam actuar como reservatório de TBT a longo prazo. Os resultados indicam também que os níveis de TBT e de cobre neste compartimento estão significativamente correlacionados; este facto pode sugerir que ambos têm uma fonte de contaminação comum (tintas antivegetativas), todavia esta relação poderá também dever-se apenas a uma coincidência geográfica de várias fontes de contaminação. Os níveis de cobre também foram analisados nos tecidos de *N. reticulatus* para determinar se este gastrópode poderia ser utilizado como bioindicador da contaminação por cobre. Os resultados demonstraram que não existe correlação significativa entre as concentrações de cobre nos tecidos de *N. reticulatus* e as concentrações daquele metal nos sedimentos. Conclui-se então que, ao contrário do TBT, as concentrações de cobre nos tecidos de *N. reticulatus* não reflectem as concentrações ambientais e, consequentemente, este gastrópode poderá não ser um indicador adequado da contaminação por este metal.

Apesar de na literatura estar amplamente comprovado que o *imposex* em populações naturais de *N. reticulatus* constitui um efeito específico da contaminação por TBT, neste trabalho avaliou-se, com base em dados de campo, se o parasitismo por tremátodes poderia induzir ou influenciar a expressão do *imposex*. Esta foi a principal lacuna de conhecimento identificada no que diz respeito à validação deste biomarcador para biomonitorização desta poluição. De facto, o parasitismo por trematódes ocorre com frequência nos gastrópodes mas raramente é considerado nos estudos de disrupção endócrina, e nunca este aspecto foi estudado em *N. reticulatus*. Verificou-se que este parasitismo é comum em *N. reticulatus* ao longo da costa Portuguesa, tendo sido identificadas seis espécies de tremátodes parasitas: *Cardiocephalus longicollis*, *Cercaria sevillana* – a cercária de *Gynaetotyla longiintestinata*, *Diphtherostomum brusinae*, *Himasthla quissetensis*, *Lepocreadium album* e uma cercária desconhecida, pertencente à família Zoogonidae. Comprovou-se que algumas destas espécies infectam o complexo glândula digestiva/gónada e podem provocar a castração dos animais, podendo ter um impacto importante na dinâmica populacional deste gastrópode. Verificou-se, ainda, que não havia diferenças entre os níveis de *imposex* expressos pelas fêmeas parasitadas e não parasitadas, levando à conclusão que o parasitismo não tem influência na expressão do *imposex*. Contudo, nos machos, o parasitismo tem um efeito redutor no tamanho do pénis. Esta observação vem reforçar a prática corrente de não se utilizarem animais visivelmente parasitados em estudos de *imposex*.

Keywords

Nassarius reticulatus, Imposex, Marine Pollution, Tributyltin, Triphenyltin, Copper, Trematodes Parasites.

Abstract

The main objective of the present work was to study the spatial and temporal evolution of TBT pollution along the Portuguese coast, using the gastropod *Nassarius reticulatus* as a bioindicator. The assessment of TBT pollution levels was essentially based on the measurement of the masculinisation degree of *N. reticulatus* females (imposex), for several sampling stations and dates, since imposex is considered a specific biomarker of TBT contamination. To assess the real dimension of this problem in Portugal, imposex was surveyed throughout the Portuguese continental coast, including the main estuarine systems and some adjacent offshore areas up to 34 m depth. It became evident that *N. reticulatus* is an extremely valuable bioindicator due to its abundance and wide distribution along the Portuguese coast, presenting, moreover, an appropriate sensitivity to describe the existing gradients of pollution in the study area.

Other nassariid species were also sampled along the coast, namely *Nassarius pygmaeus*, *Nassarius nitidus* and *Nassarius incrassatus*. Nevertheless, these species were less abundant and it was only possible to sample a sufficient number of *N. incrassatus* specimens, for imposex studies, in some of the stations along the Portuguese coast. This bioindicator is less sensitive than *N. reticulatus* regarding the assessment of the TBT pollution levels, which, in conjunction with the poor abundance, makes this species less appropriate for monitoring programs in this coast.

The *N. reticulatus* imposex survey performed in 2006 along the shoreline showed that, in general, TBT pollution levels were still high, representing an effective risk for marine ecosystems. This problem was more critical inside or in the vicinity of harbours. In fact, in about 95% of the surveyed sites, *N. reticulatus* presented imposex levels with VDSI>0.3 showing that TBT environmental levels were higher than the Environmental Assessment Criteria defined by OSPAR, above which negative impacts can occur to species.

N. reticulatus imposex was also surveyed in deeper continental shelf areas off the region of Aveiro, between 2004 and 2006, and also off the regions of Lisbon, Setúbal and Faro in 2006. The results have shown that imposex levels and TBT body burdens in the gastropod were correlated and decreased with the distance from harbours, which seem to constitute the main pollution sources in these study areas. Despite the increasing distance from the harbours and the TBT massive dilution in deeper waters, these areas were still extensively affected by TBT pollution. In fact, VDSI values higher than 0.3 were observed in several sampling stations and relevant TBT concentrations

were detected in the gastropod tissues.

The evolution on *N. reticulatus* imposex levels was studied over the last years to assess if any change in TBT pollution along the Portuguese coast occurred recently. The levels registered in 2006 along the shoreline were compared with data obtained by other authors in the same study area in 2000 and 2003. The results showed a reduction in imposex levels between 2003 and 2006, but no changes between 2000 and 2003. A reduction of *N. reticulatus* imposex was also observed in Aveiro offshore stations when comparing the results from 2004 and 2005 with 2006. The main cause of this evolution was presumably the entry in force of the Regulation EC/782/2003 in July 2003 which forbids the application of TBT-based paints (and other organotin-based paints) in any vessel type. However, it is necessary to follow the evolution of this pollution, for which the data obtained in the current work constitute an important base-line. This aspect is significant since the International Convention on the Control of the Use of Harmful Antifouling Systems on Ships will enter in force in September 2008 and states a worldwide ban on the circulation of vessels with TBT (and other organotin) based antifouling systems.

The ban of organotins from antifouling paints and their replacement by other biocides, namely copper compounds, raised some concern regarding the possible increase of this metal in the environment. To evaluate this hypothesis, the concentration of TBT and copper in the sediments were compared between 2000 and 2006 for the same sampling sites. The results showed no significant differences in the TBT and copper levels between these years. Considering that TBT pollution in the Portuguese coast has been decreasing, according to the imposex evolution analysis, the persistence of TBT contamination in sediments suggests that sediments may act as long-term “reservoir” of this compound. These results also indicate that, in this compartment, TBT and copper were significantly correlated; this fact may suggest that both compounds have a common source of contamination (antifouling paints), however this relationship could instead be due to a geographical coincidence of the several contamination sources. The results also showed that there was no significant correlation between *N. reticulatus* copper body burden and copper sediment concentration, which probably indicates that this gastropod might not be a suitable bioindicator for copper contamination, at least using this approach.

Although it has been widely proven in literature that imposex in natural populations of *N. reticulatus* constitutes a fairly specific effect of TBT pollution, one objective of the current work was to assess if trematode parasitism could influence the imposex expression. This was the main gap identified in the literature regarding the validation of this biomarker for TBT pollution biomonitoring programs. In fact, trematode parasitism is common in gastropods but it’s rarely considered in endocrine disruption studies, and this aspect was never studied in *N. reticulatus*. The results have shown that trematode parasitism in *N. reticulatus* is a phenomenon spread along the Portuguese coast and six trematode species were identified: *Cardiocephalus longicollis*, *Cercaria sevillana* – a *Gynaetotyla longiintestinata* cercariae, *Diphtherostomum brusinae*, *Himasthla quissetensis*, *Lepocreadium album* and one unknown cercariae, belonging to the Zoogonidae family. The histological analysis showed that some of these species infect the complex digestive gland/gonad, which might have an important impact in the population dynamics of this gastropod. The results have also demonstrated no difference in imposex levels expressed by parasitized and unparasitized females leading to the conclusion that parasitism had no significant influence on the imposex expression. However, in males, parasitism has a reducing effect on penis size. This observation strengthens the current practice of discarding parasitized animals from imposex studies.

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General Introduction

1.1 As tintas antivegetativas

1.1.1 A problemática da bioincrustação

O crescimento indesejável de organismos incrustantes em superfícies submersas em meio aquático, designado por bioincrustação, constitui um problema para a indústria naval a nível mundial. A bioincrustação é uma sequência de eventos influenciada por processos químicos, físicos e biológicos. Uma superfície imersa em meio aquático como, por exemplo, os cascos das embarcações, será imediatamente coberta por determinados compostos químicos dissolvidos nesse meio, desenvolvendo uma película macromolecular. A etapa seguinte neste processo é a colonização da película macromolecular por microrganismos, esporos de algas e/ou larvas de invertebrados (Pérez *et al.*, 2006).

Torna-se, portanto, essencial a protecção de todas as superfícies imersas contra este processo de bioincrustração. Considerando especificamente o caso das embarcações, se estas não estiverem protegidas com sistemas antivegetativos, poderão acumular cerca de 150 kg de organismos por metro quadrado em menos de seis meses no mar. Num navio tanque com uma superfície subaquática de 40000 m^2, a acumulação de organismos incrustantes pode representar um acréscimo até 6000 toneladas e pode conduzir a um aumento igual ou superior a 40% no consumo de combustível, devido ao aumento de peso e da resistência ao movimento (IMO, 1999). A presença de organismos incrustantes também provoca uma diminuição da capacidade de navegabilidade da embarcação. A limpeza e tratamento dos cascos das embarcações implicam a sua permanência em doca seca, que poderá custar, para um navio de grandes dimensões, até cerca de 1 milhão de euros por dia (IMO, 1999). Por outro lado, o aumento do consumo de combustível provocado pela bioincrustração implica uma maior libertação de poluentes para o ambiente. Caso toda a frota comercial do planeta apresentasse um excesso de bioincrustação nos termos acima descritos, o consumo adicional de combustível por ano seria de 70,6 milhões de toneladas. Este aumento no consumo de combustível traduzir-se-ia numa libertação adicional de 210 milhões de toneladas de dióxido de carbono contribuindo para o aumento do aquecimento global, e de 5,6 milhões de toneladas de dióxido de enxofre, resultando no aumento de chuvas ácidas (Hunter, 2007).

1.1.2 A evolução dos sistemas antivegetativos

As tentativas para proteger os cascos das embarcações contra organismos incrustantes remontam ao tempo das antigas civilizações greco-romanas, quando os cascos eram cobertos com chumbo ou bronze (Pérez *et al.*, 2006). Durante os séculos XVII e XVIII iniciou-se o revestimento dos cascos das embarcações com cobre para combater este processo (Champ & Pugh, 1987).

As primeiras tintas antivegetativas emergiram em meados do século XIX e baseavam-se no conceito da dispersão de um elemento tóxico biocida adicionado a uma matriz (Readman, 2006). Tradicionalmente, o elemento tóxico mais utilizado era o óxido cuproso mas posteriormente foram também adicionados compostos orgânicos de mercúrio e arsénio, de forma a aumentar as propriedades biocidas das tintas (Champ & Pugh, 1987). Os pigmentos usados nestas tintas eram aplicados em contacto directo com o casco das embarcações, provocando a corrosão das suas superfícies de aço. Para ultrapassar este problema foram desenvolvidos os primários para protecção contra a corrosão. Seguiu-se o aparecimento de novos produtos, incluindo as "tintas plásticas a quente", com matrizes naturais às quais se adicionavam cobre ou outros compostos biocidas, e com o desenvolvimento da química dos materiais poliméricos surgiram as "tintas plásticas a frio", que utilizam diferentes resinas sintéticas (Almeida *et al.*, 2007). Desde então, foi desenvolvida uma grande variedade de tintas antivegetativas, de diferentes classes tecnológicas, que diferiam no custo e na eficácia. Esta última depende sobretudo do controlo da taxa de libertação do(s) biocida(s) para a água (Finnie, 2006), na qual se baseia o princípio deste tipo de tintas. Foram vários os metais pesados adicionados às tintas para lhes conferir propriedades antivegetativas e anticorrosivas. Numerosos e sucessivos desenvolvimentos deram origem, em meados da década de 60, às tintas antivegetativas com base em compostos de estanho (principalmente o tributilestanho – TBT – e em menor escala o trifenilestanho – TPT), célebres pela sua elevada eficiência e versatilidade, e que substituíram as tradicionais tintas à base de cobre (Almeida *et al.*, 2007). Estas tintas antivegetativas tinham vantagens sobre as existentes: i) maior eficácia e durabilidade dos seus efeitos, ii) prevenção da corrosão galvânica em estruturas de alumínio, iii) ampliação da gama de cores possíveis (dado que o TBT é um composto incolor) e iv) baixo custo de produção. Estas vantagens fizeram com que a utilização deste tipo de sistemas antivegetativos se generalizasse por todo o planeta em apenas uma década (de Mora, 1996).

1.1.3 Utilização dos orgnoestanhos

O estanho é essencialmente obtido a partir do mineral cassirite (SnO_2). Os compostos organoestânicos (como, por exemplo, o TBT e o TPT) são sintetizados a partir do estanho por processos indutriais inventados pelo Homem, não ocorrendo naturalmente no ambiente. Durante quase 100 anos estes compostos não foram utilizados porque inicialmente não lhes foram encontrados quaisquer aplicações comerciais (Hoch, 2001). Esta situação alterou-se por volta dos anos 40 com o aparecimento da indústria dos plásticos, particularmente com a produção de cloreto de polivinilo (PVC). O PVC degrada-se quando exposto à luz e ao calor. Para prevenir este processo adicionam-se certos derivados organoestânicos (metil-, butil- e octilestanho). Actualmente, a aplicação de organoestanhos na produção de PVC mantém-se e corresponde a cerca de 70% da sua utilização total, particularmente sob a forma de compostos mono- e di-substituídos (Hoch, 2001). Para além da sua aplicação como estabilizadores do PVC, os organoestanhos mono- e di-substituídos são também utilizados como catalisadores de reacções, em áreas tais como electrodeposição, esterificação, produção de silicones e poliuretanos (dibutil- e di-octilestanho), e na produção de vidro (cloreto de monobutilestanho) (Appel, 2004; WHO, 2006). Por sua vez, os compostos tri-substituídos, cujas propriedades biocidas foram descobertas nos anos 50 (Hoch, 2001), são muito eficazes no controlo de fungos e bactérias (Piver, 1973). O TBT foi essencialmente utilizado em sistemas antivegetativos, mas também na conservação e preservação de madeiras e pedras. Para além destas aplicações, este composto também se mostrou muito eficaz na eliminação de odores em contendores do lixo, no controlo do desenvolvimento de bactérias em ambiente hospitalar (nomeadamente *Staphylococcus aureus*), na prevenção de infecções fúngicas, tais como o desenvolvimento de bolores em instalações sanitárias, entre outros. No entanto, neste tipo de aplicação, o TBT foi gradualmente substituído por outros biocidas (Piver, 1973). O TPT, também usado como biocida em sistemas antivegetativos, embora em menor escala, é sobretudo aplicado como pesticida para protecção de produtos agrícolas, tais como batatas, bananas, entre outros géneros produzidos em larga escala. Este composto também é muito eficaz como moluscicida, utilizado para controlo de gastrópodes vectores da schistosomose humana. No seu conjunto, os tri-organoestanhos (cloreto de tributilestanho, acetato de trifenilestanho, cloreto de trifenilestanho, hidróxido de trifenilestanho, dilaurato de dibutilestanho) também são usados como insecticidas (Piver, 1973). Finalmente, os compostos organoestânicos tetra-substituídos (tetra-alquil estanho, tetrafenilestanho, tetracloreto de estanho) são usados como intermediários importantes na produção de outros compostos químicos (WHO, 2006).

1.1.4 A reutilização do cobre nas tintas antivegetativas

Como anteriormente mencionado, no início as tintas antivegetativas eram tradicionalmente preparadas utilizando o cobre como agente biocida. Com o reconhecimento da eficácia dos organoestanhos, o uso do TBT generalizou-se a partir da década de 70, tornando-se o principal biocida empregue nos sistemas antivegetativos. No entanto, o cobre continuou a ser usado como aditivo nas tintas à base de TBT, para garantir um maior espectro de actividade biocida (Haynes & Loong, 2002). Actualmente, devido à proibição do uso de TBT neste tipo de tintas, a utilização do cobre nestes produtos aumentou (Claisse & Alzieu, 1993; Voulvoulis *et al.*, 2002; Jones & Bolam, 2007; Schiff *et al.*, 2007; Srinivasan & Swain, 2007).

É importante salientar que, ao contrário do TBT que é um composto de origem exclusivamente antropogénica, não desempenhando qualquer papel biológico, o cobre é um elemento natural. As concentrações ambientais de cobre variam tipicamente entre 0,03 e 0,23 μg/L à superfície da água do mar e entre 0,20 e 30,0 μg/L em água doce (Bowen, 1985, ver Srinivasan & Swain, 2007) e é um metal essencial para a fisiologia normal da maioria dos animais, tornando-se tóxico apenas quando o organismo é incapaz de regular o seu excesso (Jones & Bolam, 2007). As fontes de cobre em meio marinho são várias, não se limitando apenas às actividades, directas ou indirectas, em torno das tintas antivegetativas (lixiviação a partir das tintas, limpeza, reparação e pintura dos cascos das embarcações). Estas fontes tanto podem ser naturais (erosão das rochas e minerais ricos em cobre) como antropogénicas (efluentes resultantes de diversas actividades, tais como águas águas residuais domésticas, exploração mineira, indústria de refinação, de produção de carvão, de produção de pesticidas, entre outras fontes) (Srinivasan & Swain, 2007).

Independentemente das inúmeras fontes de cobre para o ecossistema marinho, estima-se que, globalmente, as tintas antivegetativas são responsáveis por uma descarga de cerca de 15 x 10^6 kg de cobre por ano para os oceanos (Blossom, 2002). O aumento da utilização do cobre nas tintas antivegetativas suscitou algumas preocupações relativamente aos efeitos negativos resultantes do consequente aumento das concentrações deste elemento no meio marinho. É possível que as tintas antivegetativas com cobre venham a sofrer regulamentações ambientais similares às tintas com TBT (Srinivasan & Swain, 2007), já que a utilização deste metal nestas tintas foi revista pela maioria das entidades reguladoras destes produtos.

Recentemente, a determinação da concentração de cobre nos organismos aquáticos foi incluída nas avaliações anuais do CEMP[1] promovidas pela OSPAR[2] (OSPAR, 2007). Este tipo de estudo poderá providenciar informação sobre a evolução dos níveis de cobre no meio aquático em consequência da legislação implementada relativamente à utilização de TBT nos sistemas antivegetativos.

1.2 A poluição por TBT

1.2.1 Comportamento do TBT no meio aquático

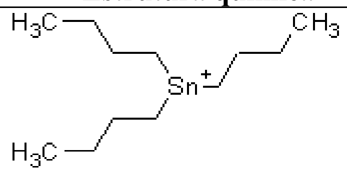
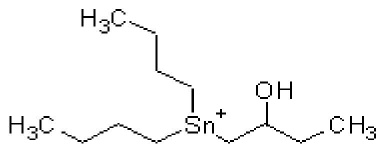
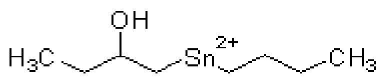
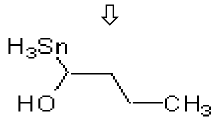
A principal fonte de TBT para o meio aquático é a lixiviação a partir das tintas antivegetativas aplicadas nas embarcações. Os compostos de TBT são derivados orgânicos do estanho (Sn^{4+}), caracterizados pela existência de ligações covalentes entre três átomos de carbono e um átomo de estanho. Estes compostos têm como fórmula química geral (n-$C_4H_9)_3$Sn-X, onde X é um anião ou um grupo covalentemente ligado e cuja natureza influencia as propriedades fisico-químicas do composto (Antizar-Ladislao, 2008).

[1] CEMP – "Coordinated Environmental Monitoring Program", coordena as monitorizações nacionais relativamente às concentrações de determinadas substâncias químicas na água, nos sedimentos e no biota e os respectivos efeitos biológicos

[2] OSPAR – Convenção Oslo e Paris para a Protecção do Meio Marinho do Atlântico Nordeste. Esta Convenção foi adoptada com vista a prevenir e combater a poluição, bem como proteger a zona marítima contra os efeitos prejudiciais de actividades humanas, salvaguardando a saúde pública, preservando os ecossistemas marinhos e, quando possível, restabelecendo as zonas marítimas que sofreram esses efeitos prejudiciais.

O destino do TBT nos ecossistemas aquáticos e as suas consequências ecotoxicológicas dependem directamente da sua persistência e, assim, da ocorrência de mecanismos de degradação. Esta degradação envolve, de uma forma geral, a remoção sequencial dos grupos butil, por desalquilação, conduzindo à formação de dibutilestanho (DBT), monobutilestanho (MBT) e estanho. A degradação do TBT, quer na coluna de água quer nos sedimentos, é influenciada por diversos parâmetros, tais como temperatura, radiação solar, matéria orgânica dissolvida, entre outros. Os mecanismos envolvidos neste processo podem ser abióticos (essencialmente a degradação química e por raios ultra-violetas) ou bióticos (principalmente degradação por bactérias e algas) (Tabela 1.1) (Maguire, 1996).

Tabela 1.1 – Degradação do TBT via desalquilação por processos biológicos.

Composto	Estrutura química	Enzima
Tribultilestanho (TBT)		
	⇩	TBT dioxigenase
β-hidroxibutil-dibutilestanho		
	⇩ Metil etil cetona +	
Dibutilestanho (DBT)		
	⇩	DBT dioxigenase
β-hidroxibutil-butilestanho		
	⇩ Metil etil cetona +	
Monobutilestanho (MBT)		
	⇩	MBT dioxigenase
β-hidroxibutilestanho		
	⇩ Metil etil cetona +	
Estanho	SnH_4	

Adaptado de "Biocatalysis/Biodegradation Database" (2008), Universidade do Minesota: http://umbbd.msi.umn.edu/

Após lixiviação a patir das tintas antivegetativas, o TBT é rapidamente removido da coluna de água devido à sua tendência para se acumular nos sedimentos. O tempo de meia-vida do TBT na água varia entre alguns dias e algumas semanas, enquanto nos sedimentos as taxas de degradação são mais baixas, resultando em tempos de meia-vida de vários meses ou vários anos (Dowson *et al.*, 1996). No entanto, a estimativa destes tempos de meia-vida baseiam-se essencialmente em experiências laboratoriais e, portanto, não reflectem as circunstâncias reais, podendo haver uma subestimação da quantidade de TBT que persiste nos sedimentos (Tessier *et al.*, 2007). Adicionalmente, sobretudo em sedimentos estuarinos, podem formar-se compostos voláteis de butilestanho metilado, que são transferidos para a coluna de água (Amouroux *et al.*, 2000; Tessier *et al.*, 2002). Point e colaboradores (2007) mostraram a existência de fluxos passivos e contínuos de butilestanhos entre a interface sedimento-água, directamente relacionados com a actividade e biomassa dos organismos bentónicos. Assim, os processos de biotransformação e de transferência modificam significativamente a mobilidade e disponibilidade do TBT nos sedimentos, enfatizando o papel dos mesmos como fonte secundária de TBT nos sistemas aquáticos.

1.2.2 Impacto do TBT nos ecossistemas

As primeiras evidências dos efeitos negativos do TBT

Os efeitos adversos do TBT no ambiente marinho foram observados pela primeira vez na Baía de Arcachon, na costa Atlântica francesa. Esta baía é particularmente conhecida pela intensa cultura de ostras, *Crassostrea gigas* (Thunberg, 1793), mas também é muito popular devido às suas marinas e numerosas embarcações de recreio. Em 1970, registou-se uma diminuição da densidade do gastrópode *Ocenebra erinacea* (Linnaeus, 1758), o que deveria ter constituído um primeiro sinal de alerta. No entanto, esta espécie era considerada uma praga em termos de ostricultura (por ser predadora de ostras) e, naquela época, a sua quase extinção não foi encarada como um problema (Santillo *et al.*, 2001). Entretanto, começaram a surgir os problemas com as próprias populações de ostras e em 1981 a sua produção anual caía de 10000 – 15000 para 3000 toneladas (Alzieu, 1991). Esta quebra na produção de ostras deveu-se a uma série de factores, desde a falha total na sua reprodução ao aparecimento de anomalias na calcificação das conchas dos animais (Alzieu, 2000). His & Robert (1985) atribuíram estes problemas à elevada concentração de TBT nas águas da baía, responsável por uma massiva mortalidade das larvas de ostras. Experiências laboratoriais e *in situ* demonstraram que concentrações de TBT acima dos 8,2 ng TBT-Sn/L afectavam significativamente o desenvolvimento normal das larvas e que acima dos 409,8 ng TBT-Sn/L afectavam a embriogénese (His & Robert, 1985).

***Os efeitos do TBT em gastrópodes – o* imposex**

Os efeitos negativos do TBT foram posteriormente registados noutros grupos de invertebrados. A presença de anomalias sexuais em moluscos neogastrópodes foi registada pela primeira vez por Blaber (1970). Este autor observou que muitas fêmeas de *Nucella lapillus* (Linnaeus, 1758) em Plymouth Sound, Reino Unido, apresentavam um pénis por detrás do tentáculo direito, apesar de serem animais com sexos separados. Nos Estados Unidos da América, em 1971, a partir da observação do gastrópode *Nassarius obsoletus (Ilyanassa obsoleta)* (Say, 1882), da costa do Connecticut, Smith (1971) descreveu o mesmo fenómeno, que designou de *imposex*, como sendo a superimposição de caracteres sexuais masculinos (pénis, vaso deferente e/ou gonoducto convolucionado) em fêmeas, parasitadas e não parasitadas, de prosobrânquios. Em 1981, o mesmo autor descobriu que havia uma relação entre o *imposex* em *N. obsoletus* e o local de colheita dos animais, tendo observado que a expressão do fenómeno era mais intensa em animais colhidos nas marinas ou nas suas proximidades e mínima ou ausente nas populações distantes daquele tipo de infra-estruturas. Colocou a hipótese de que uma substância solúvel ou em suspensão na água proveniente das marinas provocaria o fenómeno (Smith, 1981a). No mesmo ano, este autor testou uma série de compostos, usados em actividades que ocorrem nas marinas, para determinar a sua capacidade de indução do *imposex*. Duas das substâncias apresentaram esse efeito indutor e ambas eram tintas antivegetativas que continham TBT (Smith, 1981b). Ficara assim laboratorialmente confirmada a existência de uma causa ambiental para a indução do *imposex* em gastrópodes.

Anos mais tarde, em Inglaterra, Gibbs & Bryan (1986) descobriram que o fenómeno induzido pelo TBT tinha um efeito profundo na reprodução de *N. lapillus*. Neste estudo, para uma melhor descrição do fenómeno, os autores dividiram o desenvolvimento do *imposex* em 3 estádios:

- "inicial": caracterizado pelo desenvolvimento do vaso deferente e de um pequeno pénis;
- "intermédio": caracterizado pelo aumento do pénis para um tamanho próximo do pénis nos machos;
- "final": oclusão do oviducto palial pelo tecido do vaso deferente (Figura 1.1).

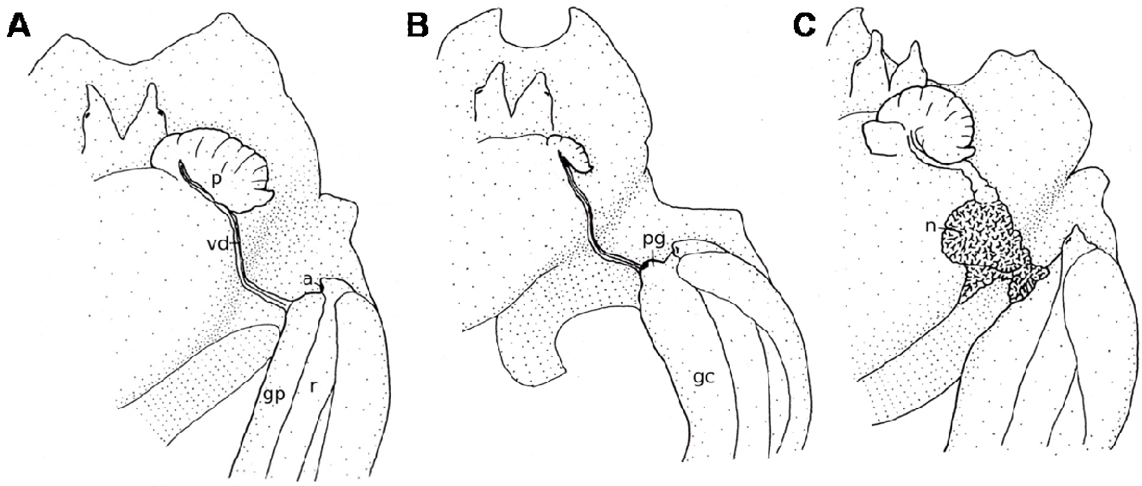


Figura 1.1 – *Nucella lapillus*. Desenvolvimento do *imposex*: (**A**) macho, (**B**) fêmea no estádio intermédio e (**C**) fêmea no estádio final: a, ânus; gc, glândula da cápsula; gp, glândula prostática; n, nódulo; p, pénis; pg, papila genital; r, recto; vd, vaso deferente. Adaptado de Gibbs & Bryan (1986).

Os dois primeiros estádios não apresentam consequências na reprodução, contudo no terceiro há o bloqueio da papila genital das fêmeas que impede a deposição de cápsulas, pelo que as fêmeas afectadas sofrem de esterilização funcional. Desta forma, demonstraram que a poluição por TBT tem um grande impacto negativo ao nível da manutenção da biodiversidade aquática.

Vários estudos se seguiram e, no início da década de 1990, Mathiessen e colaboradores (1995) referem que algumas das populações das zonas costeiras inglesas de *Littorina littorea* (Linnaeus, 1758) apresentavam um número reduzido de juvenis e falta de produção de ovos ou larvas planctónicas em quantidade. Os resultados das experiências laboratoriais realizadas por estes autores sugeriram que o TBT estava envolvido nestes efeitos. Apesar da exposição prolongada ao TBT, estes autores não observaram qualquer indicação de *imposex*, tal como o fenómeno havia sido descrito, tendo mesmo concluído que *L. littorea* era uma espécie menos sensível ao TBT comparativamente com outras, tais como *Nassarius* sp. ou *Ocinebrin*a sp. No entanto, Bauer e colaboradores (1995) observaram efeitos semelhantes na mesma espécie de gastrópodes provenientes da Alemanha e verificaram que as fêmeas de *L. littorea* expostas a TBT desenvolviam alterações ao nível do oviducto palial. Os autores denominaram esta condição de *intersex*, definido como uma perturbação na determinação fenotípica do sexo entre a gónada e o tracto genital (Bauer *et al.*, 1995).

De entre os organoestanhos, o TBT é considerado o principal responsável pela indução do *imposex* e *intersex*. São vários os estudos laboratoriais que confirmam a indução do *imposex* por exposição ao TBT em diversas espécies de gastrópodes, nomeadamente *N. lapillus* (Bryan *et al.*, 1987; Bryan *et al.*, 1988; Evans *et al.*, 2000), *Buccinum undatum* (Linnaeus, 1758) (Mensink *et al.*, 1996; Mensink *et al.*, 2002), *Thais clavigera* (Küster, 1860) (Horiguchi *et al.*, 1997), *Hydrobia ulvae* (Pennant, 1777) (Schulte-Oehlmann *et al.*, 1998), *Nassarius reticulatus* (Linnaeus, 1758) (Bettin *et al.*, 1996; Barroso *et al.*, 2002a), *Bolinus brandaris* (Linnaeus, 1758) (Santos *et al.*, 2006) e *Stratomita haemastoma* (Linnaeus, 1758) (Limaverde *et al.*, 2007). No entanto, outros organoestanhos têm a capacidade de induzir o *imposex*. Na realidade, Bryan e colaboradores (1988) realizaram experiências com *N. lapillus* e verificaram que o tri-n-propilestanho (TPrT) induzia *imposex* naquela espécie, embora com efeitos menos intensos do que o TBT, mas não observaram quaisquer efeitos nas fêmeas expostas a monobutilestanho (MBT), dibutilestanho (DBT) ou trifenilestanho (TPT). Contudo, outros autores observaram que o TPT induzia *imposex* em espécies tais como *T. clavigera* (Horiguchi *et al.*, 1995; Horiguchi *et al.*, 1997), *N. reticulatus* (Barroso *et al.*, 2002a), *B. brandaris* (Santos *et al.*, 2006) e *S. haemastoma* (Limaverde *et al.*, 2007). Como já foi referido, o TPT é essencialmente usado como fungida na agricultura e, por vezes, também em tintas antivegetativas, em conjunto com o TBT. Ao longo da costa Europeia, os níveis ambientais de TPT são geralmente baixos comparativamente com os de TBT, pelo que, nesta região, considera-se que o TBT é o principal composto indutor de *imposex* em meio aquático (Barroso *et al.*, 2002a).

Os efeitos do TBT noutros grupos de organismos

O *imposex* é um fenómeno que já foi descrito em mais de 160 espécies de gastrópodes em todo o mundo (Shi *et al.*, 2005; Horiguchi *et al.*, 2006). Para além dos efeitos negativos já mencionados em moluscos, o TBT também tem impacto noutros grupos de organismos, tais como:

- diminuição na produção primária e na concentração de clorofila *a* no fitoplâncton (Petersen & Gustavson, 2000);
- redução de processos metabólicos como a actividade fotossintética, respiração e crescimento em algas marinhas (Jensen *et al.*, 2004);
- redução do crescimento em bactérias (Mendo *et al.*, 2003);
- redução da capacidade de reprodução em crustáceos (Aono & Takuchi, 2008);
- inibição da gametogénese em peixes (Zhang *et al.*, 2007);
- acumulação no fígado e rins de peixes, aves e mamíferos marinhos (Berge *et al.*, 2004; Veltman *et al.*, 2006; Hartford *et al.*, 2007; Nakayama *et al.*, 2007), podendo provocar imunossupressão, que por sua vez pode estar associada ao aumento da prevalência de doenças nas populações selvagens (Shimasaki *et al.*, 2006).

Portanto, os efeitos do TBT não se restringem apenas a um grupo específico de organismos mas fazem-se sentir a vários níveis dos ecossistemas aquáticos, o que demonstra a necessidade de se implementar legislação para reduzir as descargas deste composto para o meio aquático.

1.3 A legislação associada à utilização de organoestanhos em tintas antivegetativas

As primeiras restrições legislativas na utilização de antivegetativos à base de organoestanhos surgiram em 1982. Devido aos problemas na baía de Arcachon, a 19 de Janeiro de 1982, o Ministro do Ambiente do governo francês anunciou uma proibição temporária (por um período renovável de 3 meses) na utilização de antivegetativos com mais de 3% de TBT, em embarcações com comprimento inferior a 25 m, quer na costa Atlântica, quer no Canal da Mancha. O decreto de 14 de Setembro de 1982 estendeu esta proibição a toda a área costeira e a todas as tintas antivegetativas com organoestanhos, com efeito a 1 de Outubro do mesmo ano (Alzieu, 2000). Todavia, esta regulamentação permitia a aplicação dessas mesmas tintas em embarcações com comprimento superior a 25 m, e em embarcações com cascos de alumínio ou liga de alumínio (Champ, 2000). Em 1985, o Ministro do Ambiente do Reino Unido anunciou a sua primeira acção reguladora com o intuito de reduzir o impacto ambiental provocado pelos organoestanhos das tintas antivegetativas. Essa acção consistiu em i) desenvolver legislação para controlo da venda a retalho das tintas com organoestanhos; ii) estabelecimento de um esquema de notificação para todos os novos agentes antivegetativos; iii) desenvolver directrizes para a limpeza e pintura de barcos com revestimento antivegetativo; iv) estabelecimento de objectivos de qualidade ambiental (EQT[3]) para a concentração de TBT na água (inicialmente foi proposto um EQT de 8,2 ng TBT-Sn/L) e v) coordenar e desenvolver programas de pesquisa e monitorização da poluição por organoestanhos para que o Governo pudesse avaliar a eficácia da implementação de nova legislação. Mais tarde, em 1987, dado que não se estava a obter a redução da poluição para níveis aceitáveis, foi implementada a proibição total da utilização de TBT em embarcações de comprimento inferior a 25 m e o EQT é reduzido de 8,2 ng TBT-Sn/L para 0,8 ng TBT-Sn/L (Champ, 2000). Os Estados Unidos da América proibiram as tintas com TBT em embarcações com comprimento inferior a 25 m em 1988 e estabeleceram uma taxa máxima de lixiviação de 4 $\mu g/cm^2$/dia para embarcações com comprimento superior a 25 m. As tintas antivegetativas com TBT também foram proibidas noutros países tais como Austrália em 1988 e Canadá em 1989 (Alzieu, 1998).

Na Europa, a Comissão Europeia propôs a restrição da venda e uso de substâncias perigosas em Fevereiro de 1988. A Directiva 89/677/CEE do Conselho, de 21 de Dezembro de 1989, alterou pela oitava vez a Directiva 76/769/CEE relativa a aproximação das disposições legislativas, regulamentares e administrativas dos Estados-Membros respeitantes à limitação da colocação no mercado e da utilização de algumas substâncias e preparações perigosas, onde se incluem os organoestanhos. Deixa de ser permitida a aplicação destes compostos em preparados utilizados como antivegetativos em determinado tipo de estruturas, nomeadamente em cascos de embarcações com menos 25 m de comprimento, em qualquer tipo de dispositivo ou equipamento usados em piscicultura e conquicultura e em dispositivos ou equipamentos total ou parcialmente

[3] EQT – "Environmental Quality Target"

submersos. Ao mesmo tempo, a utilização de organoestanhos também é proibida em preparados destinados ao tratamento de águas industriais, independentemente da sua aplicação.

O Comité de Protecção do Meio Marinho (MEPC[4]) da IMO[5] reviu a posição dos organoestanhos na sua lista de substâncias perigosas e recolheu informação sobre os efeitos destas substâncias no meio marinho e na saúde pública. Em Novembro de 1990, o MEPC emitiu uma série de recomendações relativamente à aplicação de TBT nas tintas antivegetativas, incluindo a eliminação da utilização de tintas com TBT em qualquer tipo de embarcação inferior a 25 m de comprimento, a redução das descargas de TBT nos estaleiros, a eliminação da utilização de tintas com uma taxa de libertação média superior a 4 $\mu g/cm^2$/dia e o desenvolvimento de sistemas alternativos (Champ, 2000). A maioria dos países ocidentais, incluindo a então designada Comunidade Económica Europeia (CEE), adoptou estas recomendações na sua legislação. Permaneceu ainda a questão de quão eficazes seriam estas recomendações, isto é, a proibição do TBT apenas em embarcações inferiores a 25 m de comprimento. Um estudo extensivo realizado em França demonstrou que a redução da concentração de TBT no ambiente, registada na década de 1980, tinha cessado (Michel & Averty, 1999). De facto, quando em 1982 surgiu a proibição de antivegetativos com TBT em embarcações inferiores a 25 m, a concentração do composto ao longo da costa Francesa diminuiu substancialmente no decorrer dessa década. Depois, a situação estabilizou, tendo as concentrações de TBT permanecido praticamente idênticas entre 1992 e 1997. No entanto, as concentrações apresentadas no estudo ainda permaneciam demasiado elevadas, indicando a persistência do problema, apesar dos regulamentos e recomendações adoptados. Este facto poderia dever-se à falta de sanções legais para os casos de incumprimento, contudo enquanto fosse permitida a aplicação de tintas à base de TBT em embarcações de grande porte (comprimento superior a 25 m) não haveria diminuição das concentrações deste organoestanho para níveis negligenciáveis (abaixo de 0,4 ng TBT-Sn/L), dado que a descarga deste composto para o meio aquático não havia cessado. No entanto, alguns países implementaram a proibição total da utilização deste tipo de tintas em 1990, tais como o Japão, a Suíça e a Áustria.

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[4] MEPC – "Marine Environmental Protection Committee"

[5] IMO – "International Maritime Organization", agência especializada das Nações Unidas criada em 1948 e cuja principal função é desenvolver e adoptar tratados e outros regulamentos para melhorar a segurança do tráfego naval e evitar a poluição dos oceanos.

Constatando que a legislação adoptada não surtira efeito na redução dos níveis de poluição por TBT, em Novembro de 1998 o MEPC propôs a implementação da proibição total na utilização de organoestanhos na constituição dos sistemas antivegetativos. Foi proposta a proibição de toda e qualquer aplicação de tintas antivegetativas à base de TBT a partir de 1 de Janeiro de 2003 e a proibição global da circulação de embarcações com esse tipo de sistemas após 1 de Janeiro de 2008 (MEPC, 1998). Em Outubro de 2001, em Londres, a IMO aprovou a “International Convention on the Control of Harmful Anti-Fouling Systems for Ships” (Convenção AFS) durante a “International Conference on the Control of Harmful Anti-Fouling Systems”. Esta convenção incluiu a proposta de proibição de antivegetativos com TBT e outros organoestanhos apresentada pelo MEPC. Esta proibição deveria entrar em vigor 12 meses após a ratificação por 25 Estados Membros representativos de 25% da tonelagem mercante mundial (MEPC, 2001).

Em 2002, o tratado não fora validado por nenhuma das nações signatárias iniciais. Antecipando um processo de ratificação pouco célere e ciente da necessidade urgente em implementar medidas restritivas, a Comissão Europeia adoptou as directrizes da Convenção AFS independentemente de não haver uma data prevista para a sua entrada em vigor. A Directiva 2002/62/CE da Comissão de 9 de Julho de 2002 adaptou, pela nona vez, o anexo I da Directiva 76/769/CEE do Conselho, apelando para uma revisão das disposições no que se refere aos organoestanhos utilizados nos produtos antivegetativos na sequência dos trabalhos da IMO. Mais tarde, em Abril de 2003, foi adoptado o Regulamento CE/782/2003, relativo à proibição dos compostos organoestânicos nos navios. Numa primeira fase, estas novas directrizes previam que as embarcações arvorando pavilhão de um dos Estados Membros da União Europeia (UE) não poderiam aplicar novos revestimentos contendo organoestanhos a partir de 1 de Julho de 2003 ou, no máximo, 3 meses após a publicação do regulamento em jornal oficial. No entanto, até à entrada em vigor da Convenção AFS, as embarcações que arvorassem pavilhão de outros Estados não estariam sujeitas a esta proibição. Na segunda fase, a partir de 1 de Janeiro de 2008, todas as embarcações pertencentes a um Estado Membro teriam que remover dos cascos das suas embarcações todas as tintas com organoestanhos ou então deveriam selar adequadamente as superfícies pintadas com estas tintas para impedir o contacto dos antivegetativos com a água. Estas regras também seriam aplicadas a qualquer embarcação não registada na UE que entrasse num porto ou terminal "offshore" de um Estado Membro, não obstante a Convenção AFS não ter entrado em vigor (ENDS, 2003).

A 17 de Setembro de 2007, o Panamá assinou a Convenção AFS e ficaram reunidos os requisitos necessários para a entrada em vigor da mesma. O número de estados que ratificaram a Convenção AFS subiu para 25, com uma percentagem combinada de 38,1% da arqueação da frota mercante mundial. Consequentemente, a Convenção entrou em vigor em 17 de Setembro de 2008 (IMO, 2007). Assim, a partir desta data não é permitida a aplicação, reaplicação ou circulação de organoestanhos nos sistemas antivegetativos das embarcações.

Para além destas medidas, o acetato de fentina e o hidróxido de fentina, usados como fungicidas agroquímicos à base de TPT, receberam decisões de não inclusão sob a Directiva 91/414/CEE em 2002. Os Estados Membros tiveram de retirar do mercado até Dezembro de 2002 todos os produtos que continham estas substâncias activas e os agricultores tiveram de utilizar todo o stock até Dezembro de 2003.

1.4 A monitorização da poluição por TBT

1.4.1 A Directiva Quadro da Água

A UE requer a monitorização dos organoestanhos nas águas de todos os Estados Membros e, desta forma, o TBT foi incluído na lista prioritária da Directiva 2000/60/EC ou Directiva Quadro da Água (DQA). Esta directiva entrou em vigor em Dezembro de 2000 e reflecte a mudança na política de gestão dos recursos hídricos da União Europeia, centrando-se na protecção do ambiente. Segundo a DQA "a água não é um produto comercial como outro qualquer, mas um património que deve ser protegido, defendido e tratado como tal."

A DQA estabelece um quadro de acção da União, que inclui abordagens, objectivos, princípios e medidas comuns a todos os Estados Membros. O principal objectivo é manter, quando existente, o "estado excelente" da qualidade de água e alcançar pelo menos o "bom estado" de qualidade de todos os sistemas aquáticos até 2015. Desta forma, os Estados Membros têm de assegurar uma implementação coordenada de programas para i) evitar a deterioração e melhorar o estado dos ecossistemas aquáticos, ii) promover a sustentabilidade da utilização da água, iii) reduzir os níveis de poluição e iv) contribuir para a mitigação das inundações e das secas.

A DQA tem ainda em vista outros objectivos, nomeadamente:

- integrar e harmonizar a legislação comunitária relativa às águas, colmatando lacunas existentes;
- contribuir para atingir os objectivos de alguns acordos internacionais como a Convenção OSPAR, entre outros;
- analisar economicamente as utilizações da água e a aplicação de um regime financeiro às utilizações da água (política de tarifação da água);
- fomentar, por parte dos Estados Membros, a consulta e a participação activa de todas as partes interessadas na aplicação da DQA (www.confragi.pt).

Neste contexto, existem actualmente 33 substâncias que são classificadas como prioritárias. De entre a lista de substâncias prioritárias existem 11 consideradas como substâncias perigosas prioritárias, onde se incluem, como foi referido, os compostos de tributilestanho; essas substâncias são assim classificadas por serem tóxicas, persistentes e/ou susceptíveis de bio-acumulação. As suas emissões deverão cessar até ao ano de 2020 (Directiva 2000/60/EC).

1.4.2 O **imposex** *como biomarcador da poluição por TBT*

Desde a primeira observação de *imposex* em *Nucella lapillus*, esta espécie foi extensivamente estudada para aplicação na monitorização da poluição por TBT no Reino Unido. Estudos envolveram campanhas de monitorização de *imposex* em populações naturais, experiências no campo (por exemplo, transplantação de espécimes) e ensaios laboratoriais, tendo-se comprovado que a extensão do vaso deferente e o tamanho do pénis nas fêmeas dependiam da concentração de TBT no ambiente (Mathiessen & Gibbs, 1998).

Gibbs e colaboradores (1987) propuseram que a monitorização dos níveis de TBT no ambiente fosse feita através da medição do *imposex* em populações naturais de *N. lapillus*. Estes autores descreveram dois índices para a medição do *imposex* nesta espécie, o RPSI[6] (índice do tamanho relativo do pénis) e o VDSI[7] (índice da sequência do vaso deferente). O RPSI é definido como o volume médio do pénis das fêmeas expresso em percentagem do volume médio do pénis dos machos numa mesma população. O volume do pénis é calculado elevando ao cubo o comprimento do órgão. Nos locais que apresentam níveis de poluição muito elevados, o pénis dos machos pode apresentar-se muito deformado, com o seu contorno irregular devido a excrescências nodulares que podem sobrestimar o seu comprimento, consequentemente diminuindo o valor do RPSI e subestimando o *imposex*. Contudo, o reconhecimento dos diferentes estádios do desenvolvimento do vaso deferente constitui um método mais sensível para classificar a intensidade ou expressão do *imposex*. A sequência do vaso deferente (VDS) pode ser classificada desde o estádio 0 (fêmea normal) até ao estádio 6 (fêmea extremamente afectada - estéril). Os diferentes estádios estão ilustrados na Figura 1.2 e podem ser definidos da seguinte forma (Gibbs *et al.*, 1987):

Estádio 0 – fêmea normal, sem superimposição de caracteres sexuais masculinos; o oviducto palial termina numa abertura bem definida ou vulva, situada no ápice de uma papila genital proeminente que se projecta na cavidade do manto.

Estádio 1 – desenvolvimento da porção proximal do vaso deferente, que se inicia como uma dobra no epitélio do manto na região ventral e em direcção à papila genital.

Estádio 2 – início do desenvolvimento do pénis com a formação de um primórdio por detrás do tentáculo direito.

Estádio 3 – observação de um pequeno pénis e desenvolvimento da porção distal do vaso deferente a partir da base do pénis.

Estádio 4 – as porções proximal e distal do vaso deferente fundem-se e o pénis aumenta para um tamanho e volume que se aproximam das dimensões do pénis de um macho.

Estádio 5 – proliferação do tecido do vaso deferente sobre a papila genital – devido a esta proliferação excessiva, a vulva pode deslocar-se, contrair-se ou deixar de ser visível; podem aparecer protuberâncias em torno da papila e frequentemente desenvolvem-se "nódulos" de tecido hiperplásico.

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[6] RPSI – "Relative Penis Size Index"
[7] VDSI – "Vas Deferens Sequence Index"

Estádio 6 – o lúmen da glândula da cápsula contém material resultante das cápsulas abortadas; este material pode ser constituído por uma ou várias cápsulas comprimidas, formando uma massa translúcida ou de cor acastanhada.

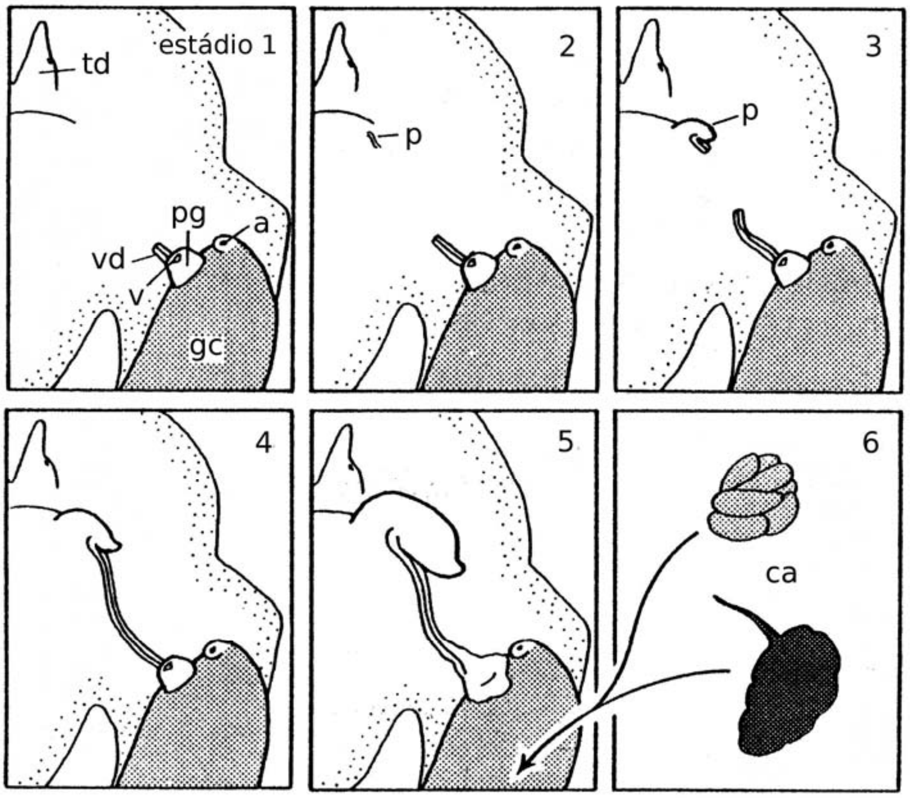

Figura 1.2 – *Nucella lapillus*. Os seis estádios utilizados para classificar a intensidade do *imposex* através da observação da sequência do vaso deferente (VDS): a, ânus; ca, cápsulas abortadas; gc, glândula da cápsula; p, pénis; pg, papila genital; td, tentáculo direito; v, vulva; vd, vaso deferente. Adaptado de Gibbs (1993).

Tal como referido anteriormente, o *imposex* encontra-se descrito para mais de 160 espécies de gastrópodes a nível mundial (Shi *et al.*, 2005; Horiguchi *et al.*, 2006). No entanto, o desenvolvimento do *imposex* nas diversas espécies nem sempre segue o esquema inicialmente proposto por Gibbs e colaboradores (1987) para *N. lapillus*. Encontram-se descritos na literatura vários esquemas de classificação da intensidade do *imposex* para cada espécie, nomeadamente: *O. erinacea* (Gibbs *et al.*, 1990), *N. reticulatus* (Stroben *et al.*, 1992a), *Hexaplex trunculus* (Linnaeus, 1758) (Axiak *et al.*, 1995), *Ocinebrina aciculata* (Lamark, 1822) (Oehlmann *et al.*, 1996), *N. incrassatus* (Oehlmann *et al.*, 1998), *Cantharus cecillei* (Philippi, 1844) (Shi *et al.*, 2005), etc. O RPSI descrito para *N. lapillus* quantifica o desenvolvimento do pénis em termos do seu volume (correspondendo ao cubo do seu comprimento), já que nesta espécie este órgão é volumoso. Noutras espécies de gastrópodes o pénis é pouco volumoso e o seu tamanho relativo, em comparação com o dos machos, é determinado através do índice do comprimento relativo do pénis (RPLI[8]= comprimento médio dos pénis nas fêmeas x 100 / comprimento médio dos pénis nos machos).

Para além destes parâmetros, em determinadas espécies é possível calcular outros índices, de acordo com as características da resposta do organismo usado como bioindicador, nomeadamente, o índice do grau de convolução do oviducto (AOS[9]) e a percentagem de fêmeas estéreis (%S). O AOS reflecte o grau de masculinização da porção gonadal do oviducto, usando um esquema de três estádios (Barreiro *et al.*, 2001). A %S está relacionada com as espécies em que o desenvolvimento do *imposex* conduz ao bloqueio da vulva, impedindo a reprodução.

O *imposex* é um biomarcador específico da poluição por TBT, que se manifesta como resposta dependente da concentração de TBT no ambiente à qual o animal esteve exposto ao longo da sua vida (Mathiessen & Gibbs, 1998). Desta forma, a avaliação do nível do *imposex* providencia informação sobre os níveis de TBT num dado local. Em alguns casos, é possível extrapolar as concentrações ambientais de TBT a partir dos níveis de *imposex*. Huet e colaboradores (1995) demonstraram a relação entre as concentrações de TBT na água do mar e os valores de *imposex* exibidos por três espécies de gastrópodes, *N. lapillus*, *O. erinacea* e *N. reticulatus*. Devido a esta especificidade, o *imposex* tem sido utilizado como ferramenta para avaliação dos níveis de poluição por TBT em todo o mundo.

1.4.3 **Nassarius reticulatus** *como bioindicador*

Nassarius reticulatus é um gastrópode prosobrânquio que apresenta uma vasta distribuição geográfica ao longo da costa Europeia (Graham, 1988). Esta espécie foi

[8] RPLI – "Relative Penis Length Index"
[9] AOS – "Average Oviduct Stage"

proposta como bioindicadora da poluição por TBT por Stroben e colaboradores (1992a). Considerando que as populações de *N. lapillus* estão ausentes em determinadas zonas do Atlântico Nordeste, a OSPAR recomenda a utilização de outras espécies para a monitorização do *imposex*, nomeadamente, *N. reticulatus* (OSPAR, 2003). Barroso e colaboradores (2002b) e Sousa e colaboradores (2005) demonstraram que este gastrópode é comum ao longo da costa ocidental portuguesa. Esta espécie é menos sensível ao TBT mas exibe um desenvolvimento de *imposex* comparável ao de *N. lapillus* (Stroben *et al.*, 1992b). A classificação do VDSI nesta espécie difere de *N. lapillus* dado que *N. reticulatus* apresenta duas vias possíveis de desenvolvimento de *imposex*, uma com a formação de um pénis (via *a*) e outra sem a formação do pénis (via *b*). Os diferentes estádios da VDS em *N. reticulatus* estão ilustrados na Figura 1.3 e caracterizam-se do seguinte modo (Stroben *et al.*, 1992a):

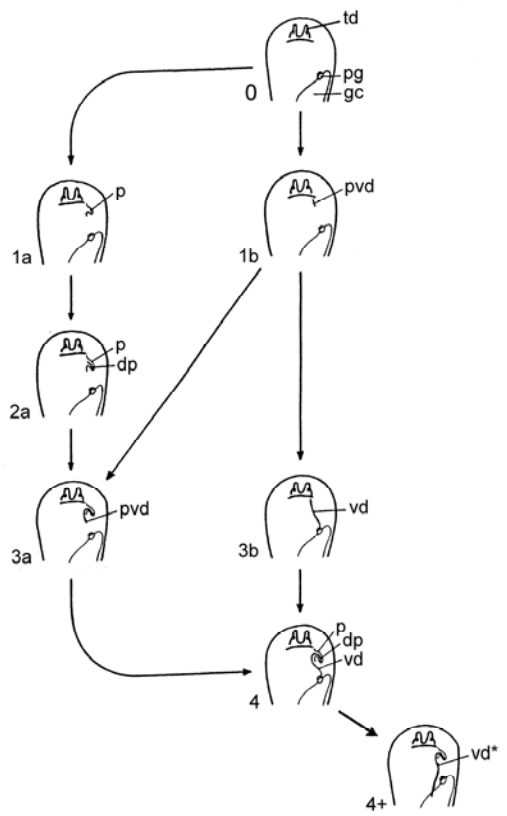

Figura 1.3 – *Nassarius reticulatus*. Esquema do desenvolvimento do *imposex*: dp, ducto do pénis; gc, glândula da cápsula; p, pénis; pg, papila genital; pvd, porção do vaso deferente; td, tentáculo direito; vd, vaso deferente; vd*, vaso deferente que se estende até à glândula da cápsula. Adaptado de Stroben *et al.* (1992a).

Estádio 1 – via *a*: pénis pequeno sem ducto, que se desenvolve por detrás do tentáculo direito; via *b*: sem desenvolvimento de pénis mas com desenvolvimento distal do vaso deferente, que se inicia por detrás do tentáculo direito.

Estádio 2 – (apenas via *a*) pénis com ducto.

Estádio 3 – via *a*: pénis com ducto que se prolonga num vaso deferente distal incompleto, que cresce sucessivamente em direcção à vulva; via *b*: sem pénis, vaso deferente estende-se desde o tentáculo direito até à vulva.

Estádio 4 – pénis com ducto; vaso deferente que se estende do pénis à vulva.

Estádio 4^{+} – o vaso deferente passa a vulva e estende-se até ao canal ventral da glândula da cápsula.

Barroso e colaboradores (2002b) propuseram uma forma alternativa de calcular o VDSI em *N. reticulatus*: em vez de atribuirem o valor numérico 4 aos estádios 4 e 4^{+} ($VDSI_4$) (Stroben *et al.*, 1992b), atribuíram o valor 4 ao estádio 4 e o valor 5 ao estádio 4^{+} ($VDSI_5$). Estes autores descrevem que a utilização desta forma de cálculo resulta num aumento do poder de discriminação dos níveis de poluição por TBT entre diferentes locais e datas de amostragem.

1.4.4 Monitorização sob a coordenação da OSPAR

O TBT está incluído na lista das substâncias químicas de acção prioritária da OSPAR (OSPAR, 2004), tendo-se tornado um elemento mandatário nos programas de monitorização desta comissão. Muitas das substâncias incluídas nesta lista são comuns à das substâncias da DQA. A diferença entre ambas as listas deve-se ao facto da DQA e a OSPAR terem usado critérios de selecção e prioridades ligeiramente diferentes para reflectir a ocorrência de substâncias perigosas no ambiente. Para monitorização dos efeitos específicos provocados por essas substâncias, a OSPAR adoptou as directrizes do JAMP[10]. Também desenvolveu os "Provisional JAMP Assessment Criteria for TBT – Specific Biological Effects" que representam os critérios de avaliação ambiental com base nos dados resultantes da monitorização dos efeitos biológicos específicos do TBT, realizada através do CEMP. Estes critérios foram desenvolvidos tendo em conta os objectivos da

[10] JAMP – "Joint Assessment and Monitoring Program", base de trabalho para alcançar os objectivos da Convenção OSPAR

Estratégia para as Substâncias Perigosas (HSS[11]) no âmbito da OSPAR (OSPAR, 2003), os Critérios de Avaliação Ambiental (EAC[12]) para o TBT na água (0,004 – 0,04 ng TBT-Sn/L), nos sedimentos (0,002 – 0,02 ng TBT-Sn/g ps[13]) e no biota (mexilhão: 0,4 – 4,0 ng TBT-Sn/g ps) (OSPAR, 1997) e ainda o desenvolvimento de Objectivos de Qualidade Ecológica (EcoQO[14]) para o *imposex* em *N. lapillus*. Estes critérios foram estabelecidos em termos de intervalos de VDSI em *N. lapillus*, que representa a espécie mais sensível à exposição ao TBT de entre as recomendadas pelas directrizes de monitorização da OSPAR, e que estão esquematizados na Tabela 1.2.

Tabela 1.2 – Interpretação das classes de avaliação referentes a *Nucella lapillus*. Este gastrópode representa a espécie mais sensível à poluição por TBT utilizada nas directrizes de monitorização no âmbito da OSPAR.

Classes de avaliação	**VDSI *em Nucella***	**Efeitos e impactos**
A	<0,3	O nível de *imposex* em espécies de gastrópodes mais sensíveis é próximo de zero (0 - ~30% das fêmeas apresentam *imposex*) indicando uma exposição a concentrações de TBT próximas de zero, que é o objectivo da Estratégia para o controlo de Substâncias Perigosas no âmbito da OSPAR.
B	0,3 - <2,0	O nível de *imposex* em espécies de gastrópodes mais sensíveis (~30 - ~100 % das fêmeas apresentam *imposex*) indica uma exposição a concentrações de TBT no ambiente abaixo do EAC. É pouco provável que ocorram efeitos adversos em grupos taxonómicos mais sensíveis causados pela exposição ao TBT a longo prazo.
C	2,0 - <4,0	O nível de *imposex* em espécies de gastrópodes mais sensíveis indica exposição a concentrações de TBT acima do EAC derivado para o TBT. Por exemplo, existe o risco de ocorrência de efeitos adversos, tais como redução do crescimento e do recrutamento, em grupos taxonómicos mais sensíveis, causados pela exposição ao TBT a longo prazo.
D	4,0 – 5,0	A capacidade reprodutora das populações das espécies de gastrópodes mais sensíveis, tal como *N. lapillus*, é afectada devido à presença de fêmeas estéreis, mas ainda existem algumas fêmeas capazes de se reproduzir. Existem evidências de efeitos adversos que podem ser directamente associados à exposição ao TBT.
E	> 5,0	As populações das espécies de gastrópodes mais sensíveis, tal como *N. lapillus*, são incapazes de se reproduzir. A maioria das fêmeas na população, se não todas, estão estéreis.
F	–	As populações das espécies de gastrópodes mais sensíveis, tais como *N. lapillus* e *O. aciculata*, extinguiram-se.

Adaptado de OSPAR (2004).

[11] HSS – Hazardous Substances Strategy tem como objectivo a prevenção da poluição das áreas marítimas através da redução contínua de descargas, emissões e perdas de substâncias perigosas.
[12] EAC – Environmental Assessment Criteria
[13] ps – peso seco
[14] EcoQO – Ecological Quality Objective

As outras espécies recomendadas como bioindicadoras (*N. reticulatus*, *Buccinum undatum*, *Neptunea antiqua* e *L. littorea*) foram também consideradas aquando do desenvolvimento dos critérios de avaliação dos efeitos biológicos específicos do TBT e os respectivos VDSI/ISI foram relacionados com os de *N. lapillus* (Tabela 1.3). Estas classes de avaliação desenvolvidas pela OSPAR permitem (OSPAR, 2004):

- uma classificação flexível relacionada com o EAC derivado para o TBT;
- a integração de efeitos biológicos e concentrações químicas;
- a comparação de valores VDSI/ISI entre espécies diferentes;
- a utilização de espécies diferentes com funções diferentes nas monitorizações;
- lidar com a análise da distribuição espacial.

Tabela 1.3 – Critérios de avaliação dos efeitos biológicos do TBT. Os critérios de avaliação do *imposex* em *Nucella lapillus* são apresentados com os valores correspondentes de VDSI/ISI para as populações simpátricas de outras espécies relevantes.

Classes de avaliação	***Nucella***	***Nassarius***	***Buccinum***	***Neptunea***	***Littorina***
	VDSI	VDSI	PCI[15]	VDSI	ISI
A	< 0,3	< 0,3	< 0,3	< 0,3	< 0,3
B	0,3 - <2,0			0,3 - <2,0	
C	2,0 < 4,0	0,3 < 2,0	0,3 < 2,0	2,0 – 4,0	
D	4,0 – 5,0	2,0 – 3,5	2,0 – 3,5	4,0	0,3 - < 0,5
E	>5,0	> 3,5	> 3,5		0,5 – 1,2
F	-				> 1,2

Adaptado de OSPAR (2004).

1.5 Estudos anteriores sobre a poluição por TBT em Portugal

Existem vários estudos de avaliação dos níveis de poluição por TBT ao longo da costa portuguesa. Os níveis de organoestanhos foram determinados em mexilhões (Barroso *et al.,* 2004; Sousa *et al.*, 2007), águas (de Bettencourt 1999; Barroso *et al.,* 2000) e sedimentos (de Bettencourt 1999; Barroso *et al.*, 2000; Sousa *et al.*, 2007). Também se encontram descritos os níveis de *imposex/intersex* para várias espécies, nomeadamente *N. lapillus* (Barroso *et al.*, 2000; Santos *et al.*, 2000 ; Barroso & Moreira, 2002; Santos *et al.*, 2002; Galante-Oliveira *et al.*, 2006), *N. reticulatus* (Barroso *et al.*, 2000; Pessoa *et al.*, 2001; Barroso *et al.*, 2002b; Sousa *et al.*, 2005; Sousa *et al.*, 2007), *H. ulvae* (Barroso *et*

[15] PCI – Penis Classification Index, caracterização do desenvolvimento sexual do pénis em 3 estágios

al., 2000; Génio, 2002), *L. littorea* (Barroso *et al.*, 2000) e *H. trunculus* (Vasconcelos *et al.*, 2006).

Estes estudos demonstraram a ocorrência de elevadas concentrações ambientais de TBT na costa portuguesa uma vez que as espécies indicadoras apresentaram valores de VDSI altos. Dois dos estudos mencionados anteriormente, Barroso & Moreira (2002) e Santos e colaboradores (2002), utilizando a mesma espécie indicadora *N. lapillus*, demonstraram que a legislação implementada na UE (Directiva 89/677/CEE) antes do Regulamento CE/783/2003 foi ineficaz na redução da poluição por TBT.

Os níveis de TBT descritos para a costa portuguesa são elevados e na maior parte dos estudos mencionados são superiores ao PNEC[16] (3,0 ng TBT-Sn/L) descrito pela U.S. Environmental Protection Agency (EPA, 2003) para o TBT na água. O factor de risco é elevado para a nossa costa e as espécies mais sensíveis, nomeadamente moluscos e plâncton, podem ser severamente afectadas, particularmente tendo em conta os valores de NOEL[17] (Tabela 1.4) propostos por Alzieu & Michel (1998).

Tabela 1.4 – Valores NOEL para a concentração de TBT na água propostos por Alzieu & Michel (1998).

TBT	**Efeito**
< 0,4 ng TBT-Sn/L	Aparecimento de caracteres sexuais masculinos em fêmeas de gastrópodes (*imposex*)
0,4 ng TBT-Sn/L	Interferência na divisão cellular do fitoplâncton (diatomáceas) e na reprodução do zooplâncton (mirocrustráceos, copépodes)
0,8 ng TBT-Sn/L	Aparecimento de anomalias na calcificação das conchas de *C. gigas*
8,2 ng TBT-Sn/L	Interferência na reprodução de moluscos bivalves
0,4 – 4,1 μg TBT-Sn/L	Efeitos adversos na reprodução dos peixes
0,4 – 409 μg TBT-Sn/L	Interferência no comportamento dos peixes

[16] PNEC – "Predicted No Effect Concentration", definida como a concentração do poluente abaixo da qual não se esperam efeitos adversos.
[17] NOEL – "No-Effect Levels", concentração mais elevada que não provoca efeitos detectáveis.

1.6 Mecanismos de indução do *imposex*

1.6.1 A disrupção endócrina

Os químicos disruptores endócrinos (EDC[18]) são considerados os principais agentes da disrupção endócrina. Estes químicos (quer naturais quer antropogénicos) interferem com o normal funcionamento do sistema endócrino dos animais, podendo i) simular (agonistas) os efeitos das hormonas, ii) inibir (antagonistas) os efeitos das hormonas, iii) alterar o padrão de síntese das hormonas ou iv) modificar os receptores das hormonas (Depledge & Billinghurst, 1999). O desenvolvimento do *imposex* em gastrópodes, por exposição ao TBT, é considerado um dos exemplos mais evidentes de disrupção endócrina (Matthiessen & Gibbs, 1998).

Ao longo do tempo, várias hipóteses foram postuladas tentando explicar a cadeia de eventos e processos moleculares que conduzem ao desenvolvimento do fenómeno nos gastrópodes. Féral & LeGall (1983) sugerem que o *imposex* em *O. erinacea* estaria relacionado com a acção de duas moléculas, o factor "retrogressivo" (RF[19]) e o factor morfogénico do pénis (PMF[20]). Concluíram que o TBT interferia na libertação do RF pelo gânglio cerebropleural das fêmeas. Em fêmeas não expostas, este factor impede o gânglio pedal de segregar o PMF, substância responsável pelo controlo do crescimento do pénis nos machos. Mais tarde, Oberdörster & McClellan-Green (2000) colocaram a hipótese de que os neuropeptídeos que controlam a diferenciação sexual nos moluscos poderiam ter capacidade para induzir o fenómeno em causa. De entre os quatro peptídeos estudados, sugeriram que o neuropeptídeo APGWamide poderia ser o PMF mencionado por Féral & LeGall (1983), uma vez que a sua localização coincide com o local onde o PMF é acumulado e foi o único peptídeo, de entre os estudados, capaz de induzir *imposex* em *I. obsoleta*. Santos e colaboradores (2006) demonstraram que a injecção do peptídeo APGWamide em *B. brandaris* não tem efeitos no desenvolvimento do *imposex* nesta espécie. Contudo, os autores salvaguardam que esta ausência de efeito só é relativa ao

[18] EDC – "Endocrine Disruptor Chemical"
[19] RF – "Retrogressive Factor"
[20] PMF – "Penis Morphogenetic Factor"

desenvolvimento e não à indução de *imposex*, já que as fêmeas testadas já apresentavam um pequeno pénis.

A disrupção da sinalização via esteróides também foi proposta para explicar o mecanismo do *imposex*. Nos peixes, a inibição do citocromo P450, conhecido como $P450_{arom}$ (CYP19A1, aromatase), provoca efeitos profundos na sexualidade destes vertebrados. Dado que se assumia que a bioquímica dos esteróides nos moluscos era similar à dos peixes, considerou-se que a inibição da aromatase nos gastrópodes poderia resultar em efeitos semelhantes (Mathiessen & Gibbs, 1998). Vários estudos registaram um aumento dos níveis de testosterona ou do ratio testosterona/estradiol em gastrópodes expostos a TBT em laboratório (Spooner *et al.*, 1991; Schulte-Oehlmann *et al.*, 1995; Bettin *et al.*, 1996). Numa complexa série de experiências de exposição de fêmeas de *N. lapillus* e *N. reticulatus* a várias concentrações de TBT, Bettin e colaboradores (1996) demonstraram que i) a exposição de fêmeas ao TBT (4,9 a 100 ng TBT-Sn/L) induz *imposex* em ambas as espécies e que este é acompanhado por níveis de testosterona também elevados e dependentes da dose, ii) a exposição de fêmeas a uma mistura de TBT e acetato de ciproterona (um antagonista dos receptores de androgénios) inibe o desenvolvimento de *imposex*, provando que o TBT actua via aumento dos níveis de testosterona, iii) a exposição de fêmeas a TBT juntamente com uma mistura 1:1 de 17 β estradiol e estrona também inibe o desenvolvimento do *imposex*, que foi interpretado como uma reposição da proporção normal entre androgénios e estrogénios e iv) a exposição de fêmeas a um inibidor específico da aromatase induz o *imposex*. Estes autores sugerem que em fêmeas de gastrópodes e em situações em que a testosterona apresenta níveis normais, os níveis elevados de TBT provavelmente competem com os da testosterona, inibindo a aromatase dependente do citocromo P450 e, consequentemente, impedindo a conversão de testosterona em 17-β-estradiol.

Outros estudos sugerem que a inibição da aromatase poderá não ser o mecanismo primário envolvido no *imposex* e, consequentemente, foram desenvolvidas outras propostas. Ronis & Mason (1996), num trabalho com *L. littorea*, apresentaram uma proposta alternativa à inibição da aromatase. Estes autores sugeriram que o TBT provocava *imposex* por bloqueio da conjugação da testosterona e metabolitos com enxofre, aumentando os níveis de testosterona livre nos tecidos e impedindo a sua excreção normal.

No entanto, esta experiência foi realizada com níveis extremamente elevados de TBT, o que poderá não reflectir as condições realistas de uma exposição ambiental. Santos e colaboradores (2005) expuseram *N. lapillus* a vários tratamentos para avaliar os efeitos na indução do *imposex* e nos níveis de testoterona/estradiol. O tratamento com um inibidor da $P450_{arom}$ (formestano) induziu *imposex* mas não produziu efeito na intensidade da sua expressão. Noutro tratamento, observaram que um inibidor dos receptores androgénicos (acetato de ciproterona) bloqueia a capacidade de indução do TBT. Relativamente às hormonas, os resultados deste estudo indicam que a biossíntese de estradiol livre não é afectada pela exposição das fêmeas ao TBT. Portanto, este estudo sugere o envolvimento de outros mecanismos na indução do *imposex*. Gooding & Leblanc (2001) sugerem que a esterificação da testosterona em ácidos gordos poderá ser o mecanismo de regulação dos níveis de esteróides em *I. obsoleta* e poderá representar o alvo da toxicidade do TBT. Gooding e colaboradores (2003) realizaram um estudo para determinar a interferência do TBT na esterificação da testosterona naquele gastrópode. Os resultados mostram que em animais expostos a TBT, os níveis de testosterona total (livre+esterificada) não sofrem alterações mas os níveis de testostrona livre aumentam de acordo com as concentrações a que os animais foram expostos e que a diminuição da esterificação da testosterona em gastrópodes expostos ao TBT não é causada pela inibição directa da enzima envolvida na esterificação nem pela supressão da sua expressão. Estes autores sugerem ainda que o alvo do TBT poderá ser um dos outros elementos do processo de esterificação da testosterona em ácidos gordos.

Apesar das inúmeras hipóteses postuladas, o mecanismo bioquímico que regula este fenómeno permanece ainda por decifrar. Nishikawa (2006) sugere que os receptores nucleares dos sistemas das hormonas intrínsecas são provavelmente o alvo dos disruptores endócrinos, uma vez que os seus ligandos intrínsecos são agentes lipossolúveis e de baixo peso molecular tal como os contaminantes ambientais. Segundo este autor, os invertebrados não têm receptores funcionais para os androgénios, pelo que nos gastrópodes as hormonas sexuais características dos vertebrados poderão não estar envolvidas no desenvolvimento sexual dos machos. Nishikawa *et al*. (2004) demonstraram que o TBT e o TPT se ligam eficientemente ao Receptor Retinóide X (RXR), um receptor nuclear, cujo homólogo foi clonado em *T. clavigera*. Estes autores também demonstraram que o ácido retinóico 9-*cis*, um ligando natural do RXR, induz o *imposex* nas fêmeas daquela espécie de gastrópode. Neste contexto, estes dois organoestanhos simulariam os ligandos endógenos do RXR, activando os mecanismos que, ao nível da transcrição nuclear, regulam o desenvolvimento sexual dos machos nos gastrópodes. Estes resultados sugerem então que, nas fêmeas de gastrópodes, o RXR desempenha um papel importante na indução e diferenciação dos caracteres sexuais masculinos (Nashikawa, 2006). A interacção entre as diferentes vias sugeridas (neuroendócrina, esteróide e retinóide) ainda permanece por esclarecer, já que a maioria das moléculas-alvo, potencialmente envolvidas nestes mecanismos propostos até à data, ainda não foram descritas para os moluscos (Castro *et al.*, 2007).

1.6.2 Os parasitas tremátodes como disruptores endócrinos

Apesar do mecanismo de indução do *imposex* ainda não estar esclarecido, não existem dúvidas de que o seu desenvolvimento é a expressão de uma disrupção endócrina. Contudo, a disrupção endócrina nem sempre tem uma etiologia química e pode resultar da exposição a outras condições e/ou factores ambientais adversos. Porte e colaboradores (2006) realçam o facto de que a falta de conhecimento sobre a endocrinologia dos invertebrados tem dificultado a compreensão da disrupção endócrina e dos mecanismos que envolvem os EDC. Além disso, existem estudos que relatam a observação de *imposex* em animais antes de 1960, embora com ocorrência muito rara (Cardwell & Meador, 1989; Garaventa *et al.*, 2006; Swennen & Horpet, 2008). Numa campanha de monitorização no sudeste asiático, observou-se que as fêmeas de *Cymbiola nobilis* (Lightfoot, 1786) e de *Melo melo* (Lightfoot, 1786) apresentavam um pequeno pénis e um vaso deferente, pelo que se deduziu que expressam *imposex* devido à exposição ao TBT. No entanto, surgiram dúvidas quando se verificou que todas as fêmeas destas espécies apresentavam estes caracteres sexuais masculinos em locais onde outros gastrópodes apresentavam níveis baixos ou ausentes de *imposex*. Estas observações sugeriam que naquelas espécies o *imposex* não seria induzido pelo TBT. Consequentemente, Swennen & Horpet (2008) estudaram espécimes dessas espécies colhidos na Indonésia e na Austrália antes de 1960. Todas as fêmeas examinadas apresentavam o típico pénis pequeno e o vaso deferente e tornou-se claro para estes autores que o fenómeno não era induzido pelo TBT. Concluíram que era um fenómeno natural de "pseudo-hermafroditismo". Contudo é importante salientar que espécies da mesma família (Volutidae) exibem *imposex* como resposta à exposição ao TBT. Garaventa e colaboradores (2006) registaram a ocorrência de *imposex* antes da utilização do TBT. Analisaram 55 espécimes de *H. trunculus* provenientes de vários museus e colhidos no Mar Mediterrâneo e na costa Atlântica portuguesa entre 1845 e 1930, ou seja, entre 30 a 115 anos antes da utilização do TBT. De entre estes animais, observaram que 4 fêmeas apresentavam caracteres masculinos, nomeadamente um pénis incidente por detrás do tentáculo ocular direito. Estes autores sugerem que poderão existir outros factores capazes de induzir *imposex*, nomeadamente exposição a metais pesados, alterações das condições ambientais, influência de outros compostos androgénicos e/ou infestação por parasitas, embora não se ponha em causa a validade do *imposex* para a monitorização da poluição por TBT.

O parasitismo, principalmente por trematódes, é um factor natural que ocorre com frequência no ciclo biológico dos gastrópodes e que raramente é considerado nos estudos de disrupção endócrina (Morley, 2006). Os trematódes são organismos ubíquos, quer em ambientes de água doce quer marinhos, e são muito comuns nos animais da zona intertidal. Os ciclos de vida dos parasitas trematódes são complexos e requerem um hospedeiro definitivo vertebrado, para o desenvolvimento de animais adultos sexualmente maduros, e pelo menos um hospedeiro intermediário, para o desenvolvimento dos estádios larvares (Figura 1.4). O hospedeiro intermediário primário comum a todos os trematódes é frequentemente um molusco e tipicamente um gastrópode (Hasseb & Fried, 1997).

Uma vez dentro do molusco hospedeiro, os parasitas provocam grandes alterações na sua fisiologia e imunologia. Estes animais necessitam de energia para o seu metabolismo, crescimento e multiplicação e para obter essa energia têm de se adaptar ao ambiente interno do hospedeiro, explorando os seus recursos (de Jong-Brink *et al.*, 2001). Os efeitos dos parasitas no seu hospedeiro podem ir desde pequenas variações metabólicas até alterações severas, como alocação de metabolitos secundários, alterações comportamentais, malformações da concha e destruição de tecidos (Jense *et al.*, 2006; Morley *et al.*, 2006; Van den Broeck *et al.*, 2007). Neste último caso, uma das potenciais consequências é a castração (inibição parcial ou total da gametogénese na espécie hospedeira devido à actividade ou presença física do parasita) já que os parasitas se alojam frequentemente nas gónadas (Mouritsen & Bay, 2000).

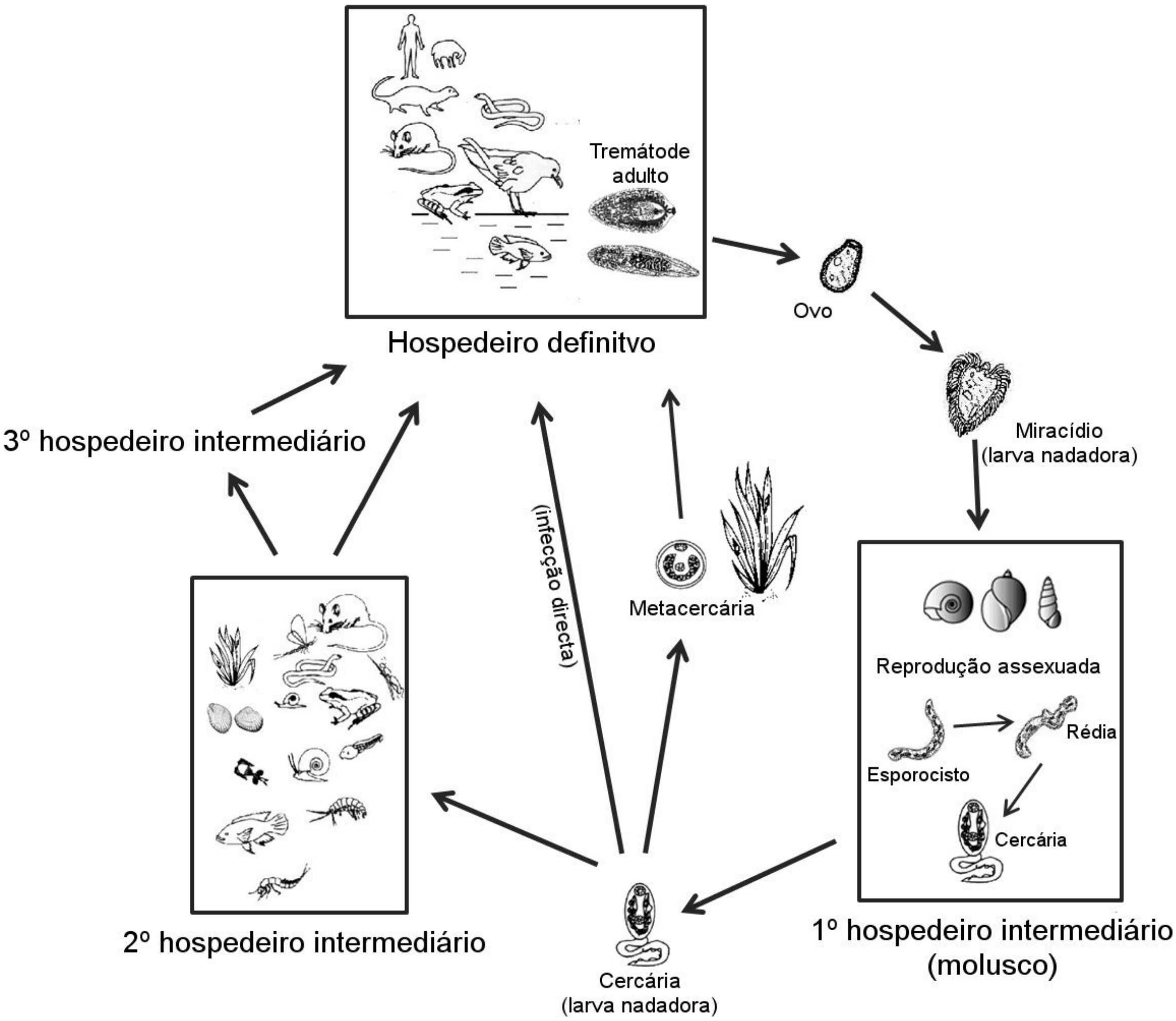

Figura 1.4 – Ciclo de vida geral dos parasitas tremátodes.

Portanto, para satisfazerem as suas exigências, os parasitas têm um enorme impacto no sistema endócrino dos moluscos podendo, teoricamente, conduzir a uma disrupção deste sistema. Poder-se-ia, então, considerar a hipótese de que estes parasitas podem interferir na expressão do *imposex* nos gastrópodes. No entanto, os poucos estudos que abordam este tema não esclarecem a interferência dos parasitas neste fenómeno.

1.7 Objectivos

1.7.1 Estudo da distribuição espacial da poluição por TBT na costa portuguesa

A poluição por TBT e o seu impacto no ambiente são temas que têm sido amplamente estudados a nível mundial. No entanto, permanecem alguns aspectos que necessitam de um estudo mais aprofundado, nomeadamente o conhecimento da real extensão da zona costeira afectada por este tipo de poluição. Sabe-se que as zonas adjacentes a portos, estaleiros e marinas são áreas bastantes afectadas pela poluição por TBT, uma vez que este composto é principalmente utilizado nas tintas antivegetativas de embarcações, as quais constituem a principal fonte de poluição para o meio aquático. No entanto, pouco se sabe sobre a evolução espacial desta poluição à medida que nos afastamos dessas fontes. Neste estudo, pretende-se avaliar a extensão da poluição por TBT em Portugal continental através da realização de campanhas de monitorização dos efeitos biológicos provocados por esta poluição – o *imposex* - no gastrópode prosobrânquio *N. reticulatus*. Pretende-se caracterizar a evolução espacial ao longo da linha de costa, entre Vila Praia de Âncora e Faro, e também em zonas mais profundas da plataforma continental (até 34 m de profundidade), ao largo de Aveiro, Lisboa, Setúbal e Faro. É importante salientar a relevância do conhecimento sobre a extensão e o impacto da poluição por organoestanhos na plataforma continental pois esta zona, para além do seu valor ecológico, apresenta tipicamente águas e fundos ricos em espécies com interesse económico, sustentando a maior parte da actividade da pesca.

1.7.2 Estudo da evolução temporal da poluição por TBT na costa portuguesa

Outro objectivo do trabalho é avaliar a eficácia da legislação em vigor na União Europeia – Regulamento CE/782/2003 – na redução da poluição por TBT. Assim, pretende-se analisar a evolução temporal dos níveis de *imposex* de *N. reticulatus* na costa continental portuguesa, não só ao longo da linha de costa (entre 2000 e 2006), como também em zonas mais profundas ao largo de Aveiro (entre 2004 e 2006). Uma vez que o *imposex* é considerado um biomarcador específico da poluição por TBT, a evolução dos níveis de *imposex* na costa portuguesa pode reflectir de forma fiável a variação dos níveis de poluição. Para completar esta análise, pretende-se também estudar a evolução dos níveis de contaminação dos sedimentos por TBT ao longo da linha de costa entre 2000 e 2006.

1.7.3 Estudo da evolução temporal da contaminação dos sedimentos por cobre na costa portuguesa

Não obstante o importante papel desempenhado pela legislação na tentativa de inverter o impacto causado pela poluição por TBT, a proibição da utilização de organoestanhos poderá estar a gerar um novo problema. Para manter as propriedades biocidas dos antivegetativos, o cobre tem sido utilizado em maior quantidade como principal componente biocida daquelas tintas. Consequentemente, a preocupação inerente ao aumento dos níveis de cobre e o seu impacto nos ecossistemas marinhos é crescente. Pretende-se, assim, estudar a evolução da contaminação dos sedimentos por cobre na costa portuguesa entre 2000 e 2006. Ao mesmo tempo, quer-se explorar a possibilidade de se utilizar o gastrópode *N. reticulatus* como bioindicador da contaminação dos sedimentos por cobre, analisando a relação entre os níveis do metal nos tecidos deste gastrópode e nos sedimentos.

1.7.4 A influência do parasitismo por tremátodes na expressão do **imposex**

Outro objectivo deste trabalho é verificar se o parasitismo por tremátodes em *N. reticulatus* pode interferir na expressão do *imposex* nesta espécie, como forma de melhor validar a utilização deste gastrópode como bioindicador. Para tal, pretende-se saber como é que as populações de *N. reticulatus* se encontram afectadas por este tipo de parasitismo na costa portuguesa, isto é, quais as espécies de tremátodes parasitas que ocorrem, qual a extensão e severidade deste problema e que patologias podem provocar no gastrópode. Finalmente, pretende-se comparar a intensidade de *imposex* em fêmeas parasitas e não parasitadas e comparar o tamanho do pénis em machos parasitados e não parasitados. Se forem registadas diferenças significativas nestes parâmetros isso significa que o parasitismo é um factor muito importante a ter em consideração em campanhas de monitorização de *imposex*. Embora seja prática corrente eliminarem-se animais visivelmente parasitados durante as campanhas de monitorização de *imposex*, o que reduz o problema, existe a possibilidade de animais em fases inicias de infecção poderem ser seleccionados para este tipo de estudos. Este facto requererá um cuidado ainda maior na selecção dos animais durante as campanhas.

1.8 Organização da tese

Esta tese está estruturada em 8 capítulos, que incluem esta introdução e uma conclusão geral do trabalho. Os outros capítulos são apresentados de seguida:

- Capítulo 2: **"Assessment of inshore/offshore tributyltin pollution gradients in the NW Portugal continental shelf using *Nassarius reticulatus* as a bioindicator"** – Realizou-se uma campanha intensiva de amostragem de *N. reticulatus*, no estuário da Ria de Aveiro (uma zona portuária importante em Portugal) e na plataforma continental adjacente a esta área (até à profundidade de 34 m), com o objectivo de i) avaliar se *N. reticulatus* poderá ser usado como espécie bioindicadora da poluição por TBT nas zonas mais profundas, ii) avaliar a magnitude e os efeitos da poluição por TBT nesta zona e iii) caracterizar os gradientes de *imposex* e organoestanhos à medida que nos afastamos das fontes destes compostos.

- Capítulo 3: **"Inshore/offshore gradients of imposex and organotin contamination in *Nassarius reticulatus* (L.) along the Portuguese coast"** – *N. reticulatus* foi colhido nas plataformas adjacentes a Lisboa e Setúbal (centro do país) e a Faro (sul do país) com o objectivo de i) combinar a determinação dos níveis de *imposex* e as concentrações de organoestanhos nos tecidos deste gastrópode para avaliar o grau de poluição nestas zonas e ii) caracterizar os gradientes à medida que nos afastamos das fontes destes compostos.

- Capítulo 4: **"Temporal evolution of imposex in *Nassarius reticulatus* (L.) along the Portuguese coast: the efficacy of the EC Regulation 782/2003"** – O principal objectivo deste estudo é avaliar a eficácia do Regulamento CE/782/2003 na redução dos níveis e poluição por TBT em Portugal, usando *N. reticulatus* como bioindicador. Os níveis de *imposex* desta espécie foram determinados ao longo da costa portuguesa em 2006 e estes resultados foram comparados com os níveis registados nos mesmos locais em 2000 e 2003, obtidos em estudos anteriores.

- Capítulo 5: **"Spatial and temporal trends of *Nassarius reticulatus* imposex on the continental shelf off Ria de Aveiro (NW Portugal): assessment of the efficacy of the Regulation EC/782/2003"** – Este estudo pretende descrever a distribuição espacial do *imposex* exibido por *N. reticulatus* em 2006, na mesma zona da plataforma continental adjacente ao estuário da Ria de Aveiro estudada em 2004 e 2005. Os resultados de 2006 foram comparados com os de 2004 e 2005, para i) analisar a utilização desta espécie na avaliação espacial e temporal da tendência dos níveis de poluição na região e ii) avaliar a eficácia da legislação internacional implementada para reduzir os níveis de poluição por TBT nas zonas mais profundas.

- Capítulo 6: **"Temporal evolution of copper and TBT contamination along the Portuguese coast between 2000 and 2006"** – O objectivo deste trabalho é a avaliação da evolução temporal entre 2000 e 2006 da contaminação por cobre e TBT nos sedimentos ao longo da costa portuguesa, atendendo à proibição total a partir de 2003, na União Europeia, da aplicação de tintas antivegetativas com TBT. Pretendeu-se também estudar a tendência temporal das concentrações de cobre nos tecidos de *N. reticulatus* no período em análise e a sua relação com os níveis deste metal nos sedimentos, para avaliar a utilização deste gastrópode como bioindicador da contaminação por cobre.

- Capítulo 7: **"Assessment of digenean parasitism in *Nassarius reticulatus* (L.) along the Portuguese coast: evaluation of possible impacts on reproduction and imposex expression"** – Os objectivos deste estudo são i) avaliar a prevalência de parasitismo em *N. reticulatus* ao longo da costa portuguesa, ii) identificar as espécies de tremátodes que infectam este gastrópode na área em estudo e iii) avaliar os efeitos dos parasitas na gónada e glândula digestiva dos animais de forma a verificar a sua interferência no desenvolvimento do *imposex* e no tamanho do pénis dos machos.

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CAPÍTULO 2
CHAPTER 2

Avaliação dos Gradientes "Inshore-Offshore" da Poluição por TBT na Plataforma Continental no NW de Portugal utilizando *Nassarius reticulatus* como Bioindicator

Assessment of Inshore-Offshore TBT Pollution Gradients in the NW Portugal Continental Shelf using *Nassarius reticulatus* as a Bioindicator

Publicado na revista científica/Published in:
"Environmental Toxicology & Chemistry" (2006) vol. 25 (12), pp. 3213 - 3220

Abstract

Imposex and organotin tissue contamination were assessed in *Nassarius reticulatus* (L.) populations in the northwest Portuguese continental shelf between 2002 and 2005 over an area of 735 km^2, involving 366 sampling sites. The objective was to evaluate the dispersion of tributyltin (TBT) from inshore sources inside the Ria de Aveiro estuary into the adjacent deeper sea and to assess endocrine disruption in the netted whelk *N. reticulatus*, using imposex as a biomarker. The highest levels of TBT tissue contamination and imposex were found in the whelks inside the Ria de Aveiro, and these declined logarithmically with distance from the mouth of this estuary. Remarkably, we found that offshore populations were also extensively affected: TBT (the dominant organotin) tissue concentration was above the detection limits at all sites where whelks were analyzed (12-356 ng TBT-Sn/g dry wt), and imposex occurred at 80% of the sampling stations, with relatively high values at some sites from the deepest area surveyed. This work shows clearly that TBT pollution is not restricted to the Ria de Aveiro but affects a significant part of the adjacent continental shelf as well. The ecological impacts of TBT pollution on offshore ecosystems are discussed.

Keywords: *Nassarius reticulatus*, Imposex, Organotins, Tributyltin, Continental Shelf

2.1 INTRODUCTION

Tributyltin (TBT) compounds have been used as biocides in ship antifouling paints since the 1960s. They leach from painted hulls into the water and accumulate in sediments and biota, causing various adverse effects on nontarget organisms. One of the most remarkable impacts is imposex — the superimposition of male characters on females — observed in a number of prosobranch molluscs (Smith, 1971). Imposex is a highly specific biomarker of TBT pollution and is manifested as a dose-dependent response throughout the animal's life, providing a robust indication of TBT exposure at a given site. Highly significant correlations have been reported between TBT concentrations in water or sediment and imposex levels in many prosobranch species throughout the world. In some cases, it is possible to extrapolate environmental TBT contamination from imposex levels, even if TBT concentrations are below limits of chemical detection. Hence, the imposex response is likely to be an essential tool for monitoring TBT pollution in offshore areas where TBT levels are generally very low because of remoteness from point sources of pollution (harbors) and to the large dilution factor.

Although levels of TBT contamination and associated impacts on inshore and estuarine ecosystems have been fairly well studied over the last two or three decades, there is still little information on comparative threats to offshore areas. Ten Hallers-Tjabbes and co-workers (ten Hallers-Tjabes *et al.*, 1994) first reported on the occurrence of imposex in offshore populations of the gastropod *Buccinum undatum,* in parts of the North Sea in 1994. Since then, there have been a limited number of observations of a similar nature for other gastropod species in the North Sea (ten Hallers-Tjabes *et al.*, 1994), the Mediterranean Sea (Chiavarini *et al.*, 2003; Gómez-Ariza *et al.* and Morabito *et al.*, in HIC-TBT, MEPC 44/INF.11 - www.nioz.nl/public/mbt/projects/hic-tbt/hic_tbt.pdf), and the Atlantic Ocean (Boon, JP in HIC-TBT, Final Report).

The netted whelk *Nassarius reticulatus* is a ubiquitous gastropod in European marine waters and has been recommended as a bioindicator of TBT pollution for the inshore maritime area covered by the Oslo and Paris (OSPAR) Convention (Joint Assessment and Monitoring Program guidelines - www.ospar.org). It has been successfully used in several TBT biomonitoring programs along European coastlines (Stroben *et al.*, 1992; Bryan *et al.*, 1993; Oehlmann *et al.*, 1993; Barreiro *et al.*, 2001; Barroso *et al.*, 2002a). In Portugal, the species is commonly found at sheltered and shallow (including intertidal) sites and is particularly abundant in estuaries (Barroso *et al.*, 2002a). Recently, we have observed that *N. reticulatus* is also very common at depths of at least 40 m off Northwest Portugal, so it is potentially a good candidate for biomonitoring TBT pollution in deeper areas of the continental shelf. To test this hypothesis, we performed intensive sampling surveys for *N. reticulatus* in the Ria de Aveiro estuary (an important natural harbor in Northwest Portugal) and the adjacent shelf down to 34 m depth, aiming to combine measurements of imposex and organotin body burden in order to evaluate the magnitude and effects of TBT pollution in the whole area and characterizing the inshore/offshore gradients.

2.2 STUDY AREA

The study area (Figure 2.1) covers 735 km^2 off the northwest coast of Portugal between Oporto (41º 08.50 N, 08º 42.59 W) and Mira (40º 32.00 N, 08º 47.03 W) with water depths up to 34 m. A major coastal feature near the centre of the study area is the Ria de Aveiro, a shallow bar-built estuary that encloses important harbors and dockyards (Figure 2.1). The topography of the Ria de Aveiro consists of three main channels that radiate from the mouth with several branches, islands and mudflats. For the most part of the Ria, the bottom is formed by sediments ranging from medium sands to muds (Cunha *et al.*, 1999). Exchange of water with the sea occurs only through the mouth, which dominates the hydrological circulation. Outside the Ria, in the area shown in Figure 2.1, the intertidal zone is a very extensive and homogeneous sandy shore. Sublittorally, the whole area consists of a relatively uniform sandy plain with a gentle slope and no obvious bottom features such as bedrock, algal beds, or biogenic structures. At shallower depths the sand tends to be finer, with the exception of a few sites close to the shore and near the mouth of the Ria, where medium sand occurs; coarser sediments (pebbly sand and sandy gravel) occur offshore, generally beyond 20 m depth (Freitas *et al.*, 2003).

Principal sources of organotin pollution in the Ria de Aveiro are the ports, dockyards and marinas. Several commercial ports and dockyards are located along the main navigation channel, which extends 9 km eastwards from the mouth to the city of

Aveiro. A deep-sea fishing port with 2 km of wharf is present as well, with more than 30 ships docked, ranging from 25 to 80 m. A large number of anchorage places and small marinas for local fishing or pleasure boats are spread along the banks of the Ria. Outside the Ria, the traffic lane for commercial ships lies parallel to the coastline, some 40 km offshore, from which an east-to-west navigational channel runs to the mouth of the estuary and shipping facilities therein. Between 4 and 6 km off the mouth of Ria de Aveiro, to the northwest, there is an anchorage site where occasionally ships may wait before entering the Ria.

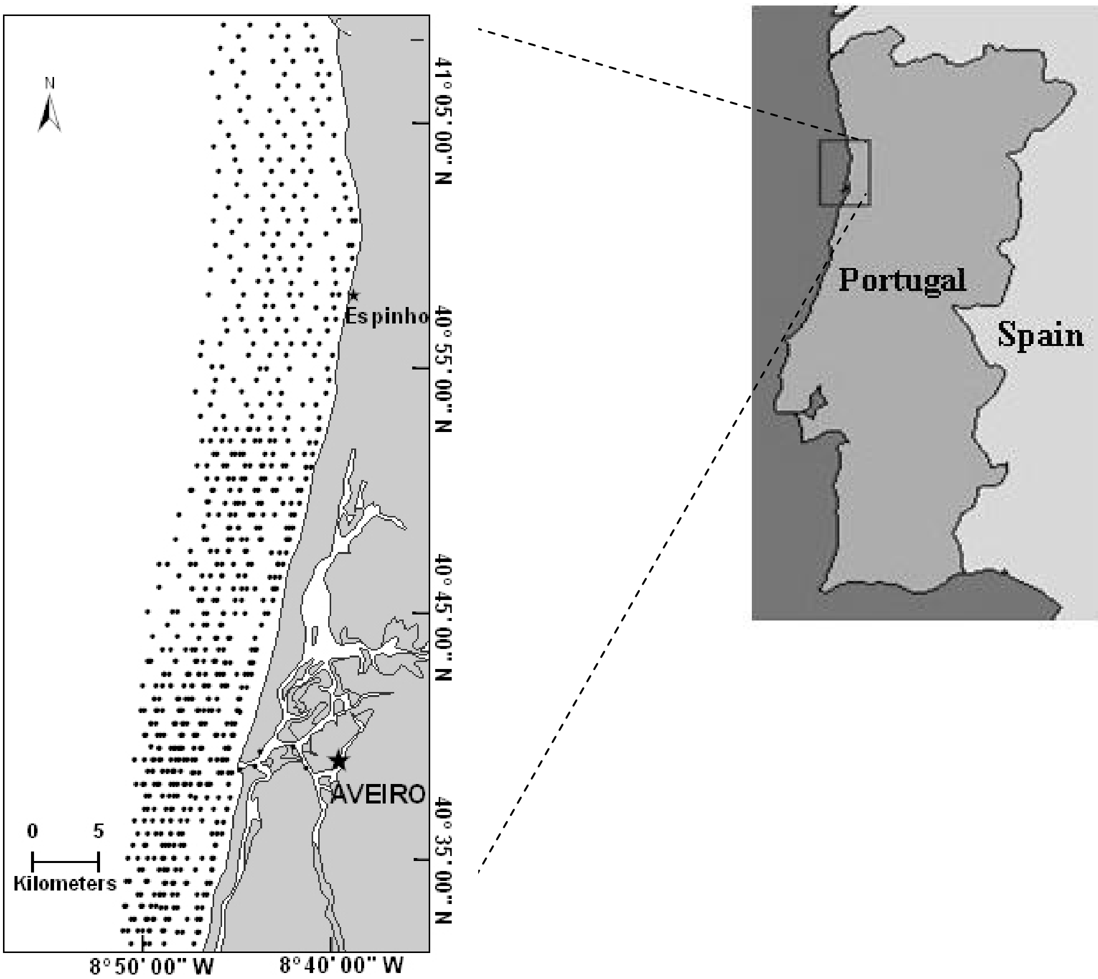

Figure 2.1 – Map showing the study area and location of the sampling sites.

2.4 MATERIALS AND METHODS

Nassarius reticulatus was sampled in 2002, 2004, and 2005 (a total of 13,276 specimens were examined). The 2002 survey took place between September 11 and October 1 and consisted of 366 sampling sites distributed along 63 transects (perpendicular to the coast) between Oporto (41º 09.00 N, 08º 46.08 W) and Aveiro (40º 38.00 N, 08º 50.11 W). The 2004 survey was undertaken between September 16 and November 30 and encompassed 226 sampling sites distributed along 43 transects between Esmoriz (40º 52.50 N, 08º 40.90 W) and Mira (40º 31.50 N, 08º 47.30 W). A third survey was performed between June 20 and 25, 2005, consisting of a total of 138 sampling sites along 23 transects between São Jacinto (40º 43.00 N, 08º 44.45 W) and Mira (40º 32.00 N, 08º 47.03 W). On each transect, between five and seven stations were situated between the bathymetric lines of 3 and 34 m. Five stations inside the Ria de Aveiro were also sampled in the 2004 and 2005 surveys.

The positioning of the sampling stations was performed using the ArcGis software from ESRI (Redland, CA, USA). At each station, sampling consisted of a 5 minutes tow using two dredges, one on each side of the boat. Each dredge was 0.64 m in width and carried a net bag of 35 mm mesh size. The total area surveyed at each site was 140 m^2. Sediment type was qualitatively assessed at each site.

In the 2002 survey the *N. reticulatus* samples were frozen immediately after collection and kept at –20ºC until imposex analysis could be carried out. However, in 2004 and 2005 the animals were maintained alive in the laboratory and examined for imposex within 3 days of collection. Animals from the 2002 survey were examined immediately after thawing, while those from the 2004 and 2005 surveys were narcotised using 7% $MgCl_2$ in distilled water. Adult *N. reticulatus* (i.e., those with white columellar callus and teeth on the outer lip) were used for imposex analysis and were randomly selected from each sample. Only samples consisting of four or more females were used. The shell height (distance from shell apex to lip of siphonal canal) was measured with Vernier callipers to the nearest 0.1 mm. Shells of *N. reticulatus* were cracked open with a bench vice and individuals were sexed and dissected under stereomicroscope. The vas deferens sequence index (VDSI) and the percentage of females affected by imposex (% I) were determined for each station. The VDSI was classified according to the scoring system proposed by

Stroben *et al.* (1992a). The penis length was also measured for the animals collected in the 2004 and 2005 surveys using a stereomicroscope with a graduated eyepiece to the nearest 0.14 mm.

A group of 66 subsamples of *N. reticulatus* females was selected from two perpendicular spatial profiles of the 2004 survey for the assessment of TBT concentrations in tissues. One profile comprised four parallel sampling transects at increasing depth, from the Ria de Aveiro mouth to the west, in order to characterize the inshore/offshore gradient of TBT tissue contamination from this point-source. The second profile comprised the deepest surveyed sites (>20 m) in a south-north direction, parallel to the coast, in order to depict any gradient in relation to the mouth of the Ria and to assess the level of contamination in the deepest zones.

The TBT tissue concentrations in *N. reticulatus* were determined using the atomic absorption spectrophotometric (AAS) methods described in Ward *et al.* (1981) and Bryan *et al.* (1986). Briefly, five or six frozen specimens were pooled and then homogenized. After acid digestion and hexane extraction, tin was measured in a PerkinElmer 76B graphite-furnace attached to a PerkinElmer 603 atomic absorption spectrometer (Wellesley, MA, USA). A 1-M sodium hydroxide solution was used to separate dibutyltin (DBT) from the TBT fraction: tin, as TBT, was subsequently measured in the NaOH-treated extracts. The recoveries for TBT were close to 100%.

As the AAS method is not a true speciation technique for organotins but rather a screening technique, a different set of 10 subsamples – collected in 2004 along a transect between the ports inside the Ria de Aveiro and offshore – were analyzed for TBT, DBT, monobutyltin (MBT), and triphenyltin (TPT) by gas chromatography/mass spectrometry (GC-MS). This transect enables a better characterization of the inshore/offshore gradients of contamination for different organotin species (including the interior of the Ria de Aveiro). The GC-MS method used is described in detail elsewhere (Szpunar *et al.*, 1996; Quintela *et al.*, 2000). In summary, two replicates of 0.1 g lyophilized tissue were taken from 15 pooled females from each sampling site and digested (microwave assisted) with tetramethylammonium hydroxide. After adjustment to pH=5, sodium tetraethylborate and isooctane, containing tetrabutyltin as an internal standard, were added successively. After microwave treatment, the organic phase was recovered and analyzed by GC-MS.

Recoveries of spiked organotins were, on average, 90 % TBT, 98 % DBT, 51 % MBT, and 98 % TPT. Throughout the current work, organotin concentrations are expressed as tin (Sn) on a dry weight (dry wt) basis; for instance, if we want to convert a given value expressed as TBT-Sn to TBT, we must multiply it by 2.44.

In order to validate and compare the results for butyltins, using the two analytical techniques (AAS and GC-MS), a mussel certified reference material, CRM 477 (Community Bureau of Reference, Commission of the European Communities, Geel, Belgium) was analyzed by each method. As there is no certified value for TPT, the reference value used was that reported by Morabito *et al.* (in HIC-TBT, MEPC 44/INF.11) for the same material. The measured organotin concentrations for three CRM replicates (mean ± standard deviation, expressed ng Sn/g dry weight) in the current work were: TBT=1,245±53 by the AAS method and TBT=750±40, DBT=660±30, MBT=986±70 and TPT=574±65 by the GC-MS method. Corresponding certified/reference values (mean and half the 95% confidence interval) were: TBT=901±77.8; DBT =785±61; MBT =1,013±189; TPT =540±140 (also expressed in ng Sn/g dry wt) indicating reasonable agreement.

Throughout this paper, statistical treatment using correlation analysis refers to the Pearson product-moment correlation coefficient (Sokal & Rohlf, 1995).

2.5 RESULTS

2.5.1 Imposex in **Nassarius reticulatus**

A total of 3,131 adult specimens were analyzed in the 2002 survey, of which 2,014 were females (64%) and 1,117 were males (36%). Females affected with imposex were spread over the area and occurred at one-third of the 72 sites where females were obtained. However, of the 122 females affected by imposex, eight possessed a well-developed vas deferens which reached the vulva (VDS stage 4), and 105 exhibited a VDS stage 3 (five of these females presented the alternative 'b-way' as described by Stroben *et al.*, 1992a). Unexpectedly, no females with VDS stage 2 were found and only nine females exhibited VDS stage 1. However, the low observed frequency of VDS stages 1 and 2 in these samples may be artefactual because of difficulty in detecting these stages in frozen and thawed specimens, leading to underestimation of the incidence of imposex in the area. In the 2004 and 2005 surveys, imposex was examined in living animals and the frequency of VDS stages 1 and 2 was higher (Figure 2.2). This indicates that imposex should be determined on living animals whenever possible.

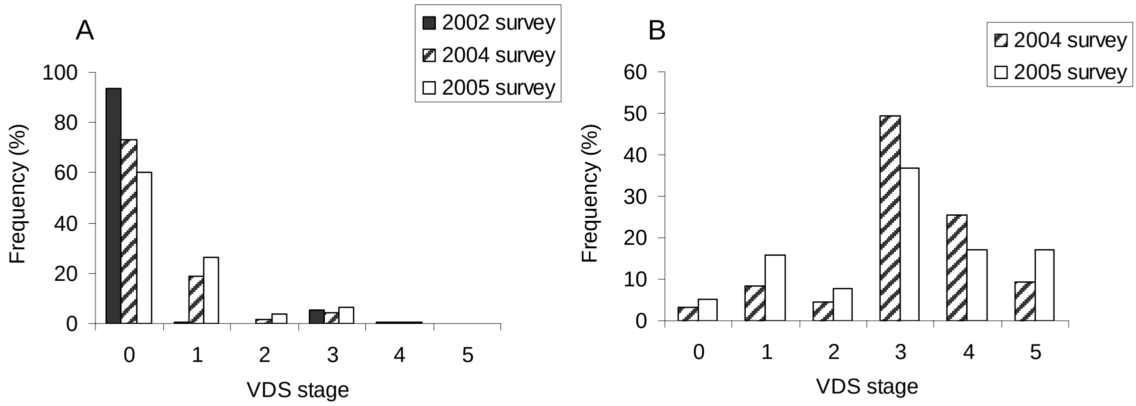

Figure 2.2 – *Nassarius reticulatus*. Individual vas deferens sequence (VDS) stage frequency observed in the females at the continental shelf off Ria de Aveiro (**A**) and inside the Ria de Aveiro (**B**) in different years.

Consequently, in the current work, we focus on the results obtained on live *N. reticulatus* in the 2004 and 2005 surveys. The number of females examined at each station was sufficient to provide robust statistics on the imposex indices per site: in the 2004 survey, 20 to 35 females were analyzed at 55% of the sites, 10 to 20 females were examined at 30% of the sites and 4 to10 females were analyzed at remaining locations (15%); in the 2005 survey, 20 to 35 females were examined at 53% of the sites, 10 to 20 females at 31% of sites and 4 to 10 females in the remaining 16% of sites.

In the 2004 survey, 6,239 adult animals were examined, of which 3,960 were females (63%) and 2,279 were males (37%). Off the Ria de Aveiro, imposex occurred in 173 of the 217 sites examined, representing an imposex incidence of 80% (Figure 2.3A). Although imposex was spread over the entire range of latitudes and depths, prevalence and severity declined logarithmically with distance from the mouth of the Ria (Figure 2.4), the main hotspot in the area, but tended to be higher to the south and west of the estuary outflow (Figure 2.3A and B). The percentage of females affected by imposex across sites varied between 0 and 100%, the VDSI ranged between 0 and 2.5 and the PLI varied from 0 to 1.1 mm. No obvious relationship was observed between the imposex indices and the type of sediment at the sampling sites, which consisted mainly of sand, pebbly sand, and sandy gravel. At sites inside the Ria de Aveiro, incidence of imposex per station varied between 93 and 100%, VDSI ranged between 2.4 and 4.3, and penis length in females between 0.4 and 16.1 mm.

In the 2005 survey, a total of 3,906 specimens were analyzed, of which 62% were females and 38% were males, again denoting an apparent predominance of females in the population. Off the Ria de Aveiro, imposex occurred at 128 of the 136 (94%) sites sampled, with a similar spatial pattern to that observed in the previous year (Figure 2.5). The incidence of imposex was higher than in 2004 because the sites most distant from the Ria (those least affected) were not sampled on this occasion. The percentage of females exhibiting imposex at each station varied between 0 and 100%, the VDS index ranged between 0 and 2.5, and the mean penis length varied from 0 to 1.2 mm. Inside the Ria de Aveiro, the same indices varied between 89 and 100%, 1.9 to 5 and 0.9 to 7.8 mm, respectively. No sterilized females were found in the current study.

The results demonstrate that imposex levels are much higher inside the Ria de Aveiro than outside. This is particularly obvious for the VDSI, which reaches the maximum score possible of 5 (see Figure 2.2) close to the ports inside the Ria. Also, the female penis length attains mean values of up to 95% of that of the males at the same locations. Outside the Ria de Aveiro, imposex is lower – VDSI never surpasses a value of 2.5 and the female penis never exceeds 15% of the male penis length. Nevertheless, the values at some of the offshore sites are much higher than expected, given the distance from point sources and the large extent of the mixing zone, signifying a widespread pollution problem in the area.

2.5.2 Organotin concentrations in **Nassarius reticulatus**

Of the 66 samples analyzed by AAS, the TBT concentrations in females varied from 12 to 356 ng TBT-Sn/g (dry wt) and, in general, decreased logarithmically with distance from the mouth of the Ria de Aveiro (Figures 2.3C and 2.4D).

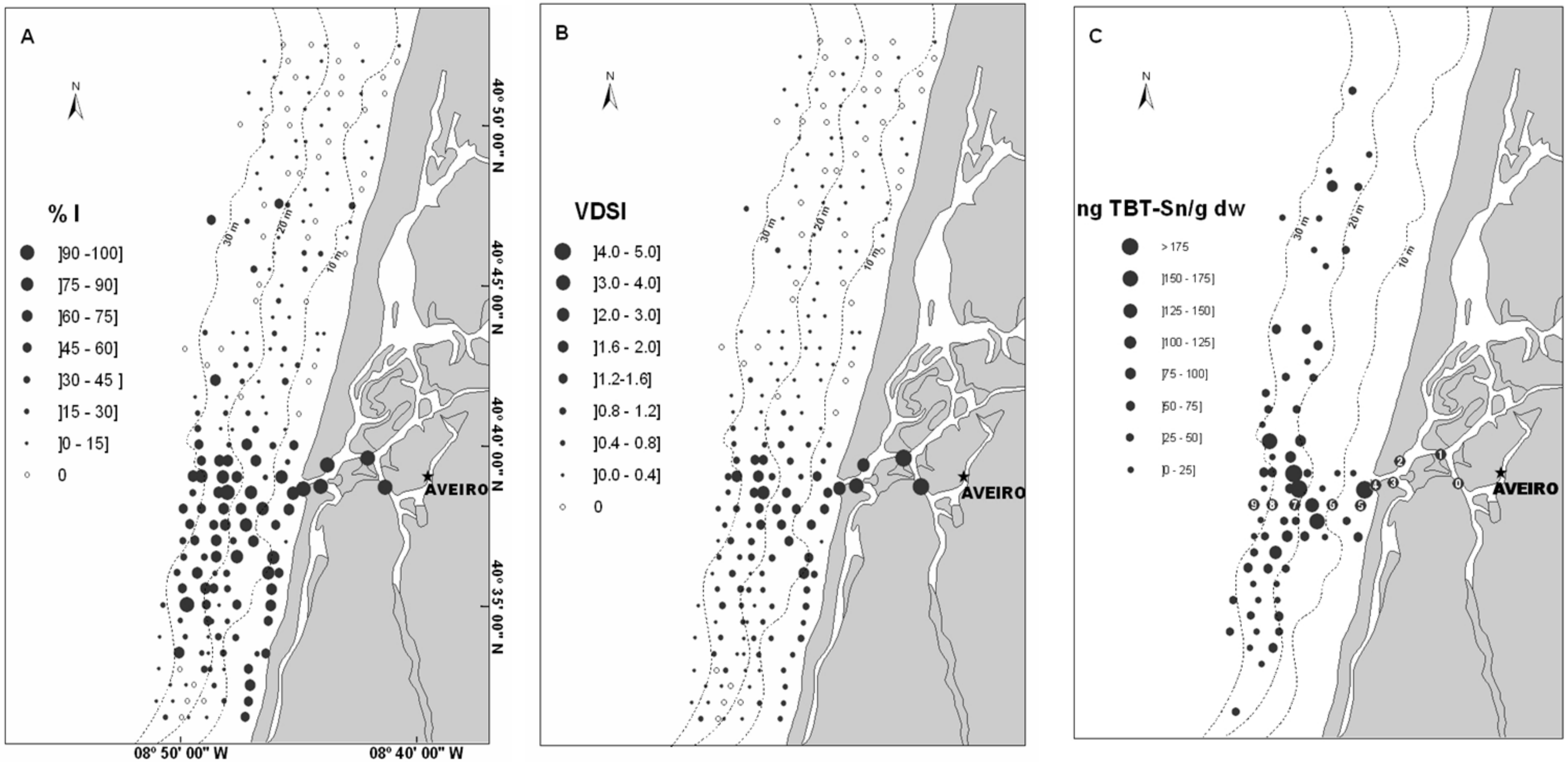

Figure 2.3 – *Nassarius reticulatus*. 2004 survey. Map of the Portuguese continental shelf between Esmoriz and Mira indicating the spatial distribution of (**A**) the imposex incidence (%I), (**B**) the vas deferens sequence index (VDSI), and (**C**) tributyltin (TBT) concentration in female whole tissues performed by atomic absorption spectrophotometry (AAS) (●) and by gas chromatography-mass spectrometry (GC-MS)(sampling sites 0-9: compare Table 2.1).

However, the decline in TBT concentrations away from the mouth of the Ria was somewhat erratic in both north-to-south and east-to-west directions (Figure 2.3C), suggesting modifying influences caused by local hydrology or secondary hotspots of TBT pollution.

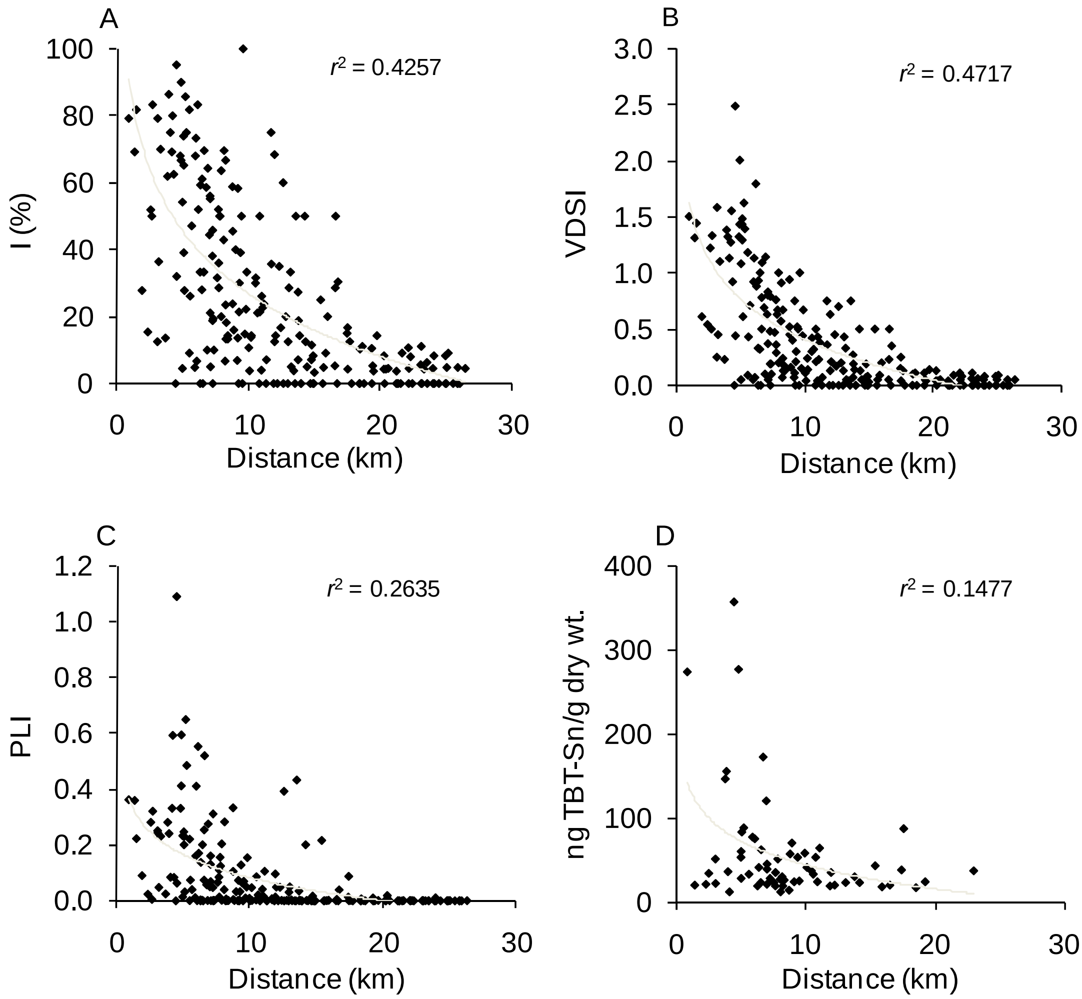

Figure 2.4 – *Nassarius reticulatus*. Correlation analysis between the distance from the mouth of the Ria and (**A**) the imposex incidence (r=0.67, P<0.001, N=217), (**B**) the vas deferens sequence index (VDSI) (r=0.69, P<0.001, N=217, (**C**) the penis length index (PLI) (r=0.51, P<0.001, N=217), and (**D**) the TBT female tissue concentrations (r=0.38, P<0.001, N=66).

The GC-MS results for butyltins across sites varied between 5 to 129 ng TBT-Sn/g dry weight, 11 to 66 ng DBT-Sn/g dry weight and <10 to 175 ng MBT-Sn/g dry weight (Table 2.1). The TBT values obtained by GC-MS tend to be slightly lower than those obtained by AAS when samples of adjacent transects are compared, a similar trend also observed in analysis of certified reference material. Mass balance estimates indicated that tributyltin represented an average of 51% of the ΣTBT+DBT body burden across stations. Triphenyltin concentrations in *N. reticulatus* were low relative to butyltins, varying from <5 to 20 ng TPT-Sn/g dry weight and were detected only inside the Ria or immediately next to the mouth of this estuarine system.

The TBT tissue concentrations were also higher inside the Ria de Aveiro (between 15 to 129 ng TBT-Sn/g dry wt) than outside (5 to 58 ng TBT-Sn/g dry wt), which is consistent with suspected estuarine sources (mainly ports and dockyards). However, our results indicate that the decrease in TBT values is not entirely uniform along the inshore/offshore transect. Values initially decline off the mouth of the Ria, slightly increase near the 20 m contour, and fall again at deeper sites. It is significant that TBT contamination in *N. reticulatus* was above detection limits in all samples analyzed, with relatively high values occurring at a number of sites, including some from the deepest area surveyed, showing that TBT pollution is not restricted to the Ria de Aveiro but affects the adjacent coastal zone as well.

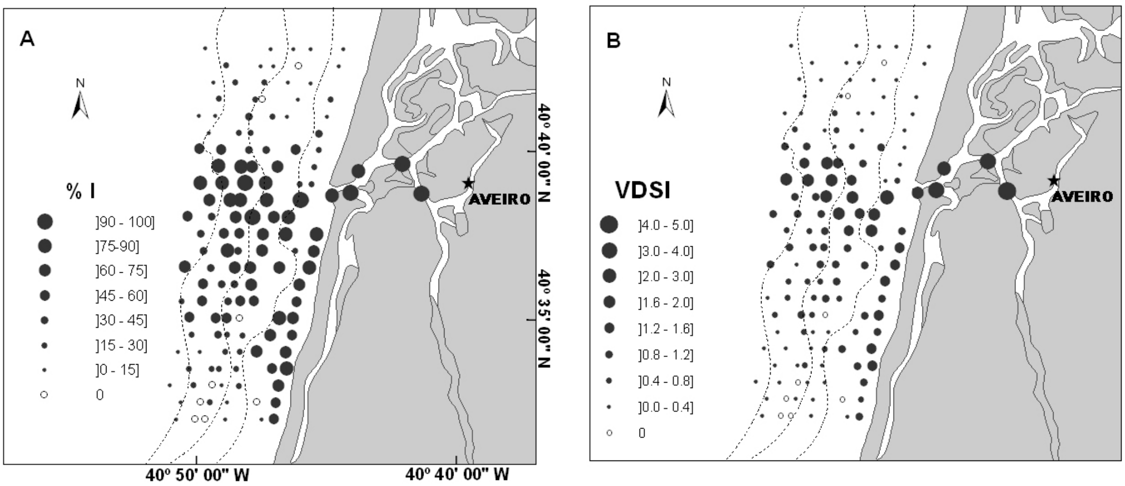

Figure 2.5 – *Nassarius reticulatus*. 2005 survey. Map of the Portuguese continental shelf between S. Jacinto and Mira indicating the spatial distribution of (**A**) the imposex incidence (%I) and (**B**) the vas deferens sequence index (VDSI).

One obvious effect of this contamination is the induction of imposex in *N. reticulatus*, which is widely spread across the entire surveyed area. Significant correlations were established between TBT concentrations in females (measured by AAS) and the incidence of imposex ($r=0.41$, $P<0.001$, $N=66$), the VDSI ($r=0.57$, $P<0.001$, $N=66$) and the PLI ($r=0.66$, $P<0.001$, $N=66$) (Figure 2.6). Similar significant correlations also occur between TBT concentrations measured by GC-MS and the VDSI ($r=0.66$, $P<0.01$, $N=10$) and the PLI ($r=0.90$, $P<0.001$, $N=10$).

Table 2.1 – *Nassarius reticulatus*. GC-MS results for organotins (ng Sn/g): tributyltin (TBT), dibutyltin (DBT), monobutyltin (MBT) and triphenyltin (TPT) (N=10). Standard deviations are given as a percentage of the mean: (a) 0 to 5%, (b) 5 to 10%, (c) 10 to 15%, (d) 15 to 20%, (e) 20 to 25% and (f) 25 to 30 %.

Station (*N*=10)	TBT ng Sn/g	DBT ng Sn/g	MBT ng Sn/g	TPT ng Sn/g
0	41^{a}	36^{c}	101^{b}	6^{d}
1	129^{a}	66^{c}	175^{c}	12^{d}
2	59^{a}	43^{b}	104^{a}	7^{a}
3	44^{b}	25^{c}	27^{d}	17^{d}
4	15^{e}	15^{b}	<10	12^{f}
5	5^{a}	14^{a}	<10	20^{f}
6	7^{a}	11^{f}	<10	<5
7	58^{b}	36^{f}	64^{d}	<5
8	37^{c}	30^{f}	13^{d}	<5
9	6^{d}	12^{b}	<10	<5

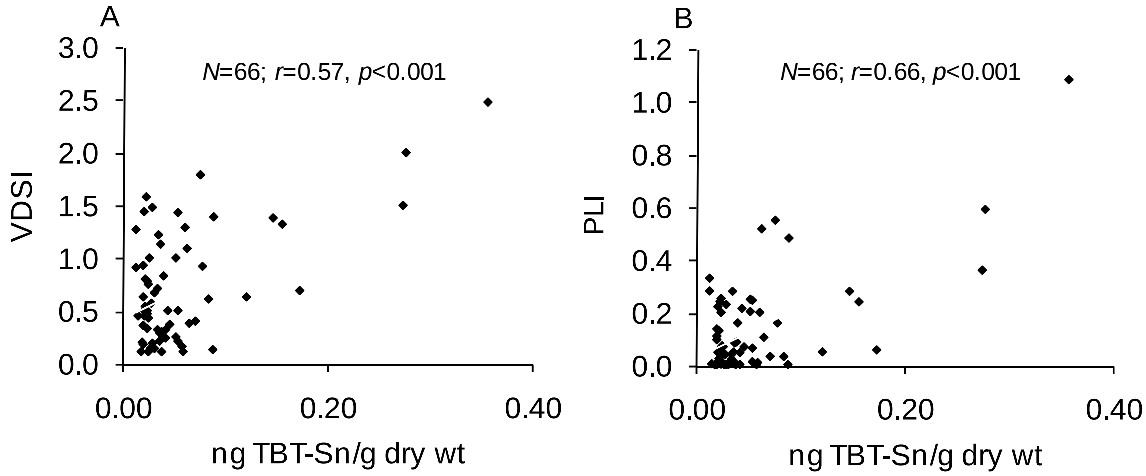

Figure 2.6 – *Nassarius reticulatus*. Correlation between the (**A**) vas deferens sequence index (VDSI) and (**B**) penis length index (PLI) with tributyltin female tissue concentration determined by atomic absorption spectrometry.

2.6 DISCUSSION

Nassarius reticulatus is a common species in the northwest Portuguese continental shelf. This fact, associated with an intense sampling effort, allowed the collection of a good number of specimens that provided valuable information for the assessment of the TBT pollution in the area.

The strong positive correlation found between TBT contamination in females and the degree of imposex development upholds the well-established concept that imposex is a fairly specific response to TBT (see review by Matthiessen and Gibbs, 1998). In the case of *N. reticulatus*, Barroso *et al.* (2002b) found, under laboratory conditions, that imposex may be induced both by TBT and TPT. Triphenyltin is a fungicide applied in agriculture but is also used in antifouling paints in a small proportion together with TBT. However, the environmental levels of TPT are generally low in comparison to TBT. In this study, TPT was detected in *N. reticulatus* only at sites inside the Ria de Aveiro or next to the mouth, representing, on average, only 21% of the combined TBT+TPT residue. Similarly, along the west Iberian coast, the relative proportion of TPT in the same species was equally low, and it was detected only at sites where TBT concentrations were high

(Barreiro *et al.*, 2001; Barroso *et al.*, 2002a). As imposex is triggered at much higher levels of TPT in comparison to TBT (Barroso *et al.*, 2002b), imposex in natural populations is mainly attributed to TBT contamination. Off the Ria de Aveiro, TBT is almost certainly the main cause of imposex, as it was the only triorganotin detected in tissues.

The spatial pattern of imposex was remarkably similar between the 2004 and the 2005 surveys, confirming previous observations that the distribution of imposex levels does not change randomly over time – a prerequisite when assessing spatial patterns of pollution. Furthermore, the imposex levels found inside the Ria de Aveiro in 2004 and 2005 match the same spatial pattern detected in this estuarine system in previous studies in 1998 (Barroso *et al.*, 2000), 2000 (Barroso *et al.*, 2002a) and 2003 (Sousa *et al.*, 2005). Thus, the highest imposex and TBT levels are found near ports and dockyards but decline substantially within 200 to 500 meters of these hotspots. Tallmark (1980) has reported seasonal migrations of *N. reticulatus* between deeper and shallower waters in the Gullmar Fjord (Sweden), which might compromise bioindicator potential; however our time-series results, based on TBT and imposex distributions, suggest that these migrations, at least in lower latitudes, must occur across very short distances.

The highest incidence of imposex and TBT contamination was observed, consistently, inside the Ria de Aveiro, where it affected most females. Severity of the condition, expressed as VDSI and PLI, was also highest here: at sites close to ports the VDSI was at or near the maximum possible score of 5 and the PLI was close to the mean male penis length. This estuarine system is clearly the dominant TBT hotspot in the study area because it embraces important dockyards and fishing/commercial ports: it has been estimated that a single TBT-antifouled ship may leach up to 1,600 ng TBT (as Sn) into the water per day per cm^2 of painted surface, whilst at anchor (Batley, 1996). There are also small boats spread around the Ria, some still using TBT coatings.

The Ria also appears to represent the most significant source of TBT pollution to adjacent coastal waters. As a consequence, the imposex levels in offshore populations of *N. reticulatus* generally reflect the pattern of decreasing TBT bioavailability with distance from the mouth of the Ria, modified partially by localized parameters. Thus, TBT pollution is somewhat more intense to the south and west, and there are some unexpectedly high values in deeper zones to the west. A number of putative causes may explain these local trends and anomalies. The residual current flow off the Ria is predominantly southward, and, in addition, on the ebb, the contaminated water from the Ria de Aveiro is pushed offshore by a jet flowing west and southwest from the mouth, which can be visually detected by airborne observations (da Silva *et al.*, 2001). This could explain the higher TBT contamination and imposex in these directions. Secondary sources of TBT pollution could also be a factor, though having minor relevance. The east-to-west navigational channel runs to the mouth of the estuary close to stations 4 to 9 (Figure 2.3). The offshore anchorage site located between 4 and 6 km to the northwest of the mouth of Ria de Aveiro is occasionally used (Figure 2.1) and may constitute a possible minor source of TBT. Dredged material consisting of contaminated sediments from the interior of the Ria de Aveiro is rarely discharged into this anchorage site (most is placed on dry land) and this may perhaps also contribute to an additional minor input of organotins to this area.

The impact of TBT pollution in the Ria de Aveiro has been identified in previous studies. Besides *N. reticulatus*, other gastropods which display high levels of imposex include *Nucella lapillus*, *Littorina littorea*, and *Hydrobia ulvae*. Notably, *N. lapillus* exhibits significant levels of female sterility at highly TBT-contaminated sites near the Aveiro ports and dockyards (Barroso *et al.*, 2000). Moreover, some oyster farms attempting to culture *Crassostrea angulata* and *C. gigas* in sheltered areas of the Ria are currently facing problems with shell growth anomalies induced by TBT contamination.

In contrast, the impacts of TBT pollution on the offshore ecosystems near the Ria de Aveiro have not been assessed previously. The current work shows that TBT pollution is undoubtedly a matter of concern in the deeper shelf since imposex in *N. reticulatus* is extensively spread in the area with VDSI levels up to 2.5. A VDSI of approximately 1 to 2.5 in this species is indicative of TBT contamination in water in the range 1 to 5 ng Sn/L (Huet *et al.*, 1995). Despite the relative ambiguity in these values, they may surpass the saltwater chronic criterion for TBT of 3 ng TBT-Sn L^{-1}, set by the U.S. Environmental Protection Agency (EPA, 2002). The no-observed-adverse effect level proposed by Alzieu and Michel (1998) implies that planktonic organisms and mollusks are likely to be the most affected: suggested levels are <0.5 ng TBT-Sn/L for imposex in gastropods, 0.5 ng TBT-Sn/L for phyto- and zooplankton growth, and 0.7 ng TBT-Sn/L for calcification anomalies in oysters (*C. gigas*). By contrast *N. reticulatus* is viewed as a moderately sensitive species: a VDSI of 1 to 2.5 in samples from European waters would equate to TBT contamination sufficient to produce imposex stages close to sterilization in other, more sensitive gastropods, such as *Ocinebrina aciculata*, *Nucella lapillus* and *Ocenebra erinacea* (Huet *et al.*, 1995; Gibbs & Bryan, 1996; Oehlmann *et al.*, 1996; Barroso *et al.*, 2002a). This comparison between species of differing TBT sensitivities should be examined in future over the northwest continental shelf area.

In conclusion, the present study shows that *N. reticulatus* populations over a large area of the northwest Portuguese continental shelf are contaminated by TBT and affected by imposex. The dispersion of TBT from the Ria de Aveiro into offshore areas may have caused widespread impacts to marine ecosystems on the continental shelf over the recent decades and undoubtedly continue to be a threat. This situation probably occurs in large areas of deeper sea in the vicinity of estuaries or inshore ports around the world and is a cause for great concern. Our results also form a substantial baseline and time series on TBT pollution in the region with which to assess the success and timescales of recovery following the International Maritime Organization's recommendation for a global ban on TBT antifouling, due to be in place by 2008.

Acknowledgement. We are most grateful to Manuel Sobral and Francisco Maia from Instituto de Investigação das Pescas e do Mar (IPIMAR) for the assistance during the sampling surveys. Part of this work was developed under research project POCI/MAR/61893/2004 and supported through a PhD grant (SFRH/BD/12441/2003) attributed by the Portuguese Foundation for Science and Technology (FCT), funded by the Portuguese Government and FEDER through the Program POCI 2010.

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CAPÍTULO 3

CHAPTER 3

Avaliação dos Gradientes "Inshore-Offshore" da Poluição por Tributilestanho na Costa Central e a Sul de Portugal utilizando *Nassarius reticulatus* (L.) como Bioindicador

Assessment of Inshore-Offshore Tributyltin Pollution Gradients in the Central and South Portuguese Continental Coast using *Nassarius reticulatus* (L.) as a Bioindicator

Publicado na revista científica/Published in:

"Marine Pollution Bulletin" (2008) vol. 56 (7), pp. 1323 - 1331

Abstract

Imposex and organotin (OT) tissue contamination of the netted whelk *Nassarius reticulatus* (L.) were assessed in the continental shelves around the main estuaries of the central coast of Portugal (Lisbon: Tagus estuary; Setúbal: Sado estuary) and the main coastal lagoon in southern Portugal (Faro: Ria Formosa). Pollution levels were higher in areas of more intense boat traffic and shipyard activities and imposex showed a clear decreasing gradient from the estuaries to the offshore, in relation to a similar gradient of tissue contamination by tributyltin. Remarkably, imposex was extensively spread over the adjacent continental shelves of Tagus and Sado estuaries. The current work shows that TBT pollution is undoubtedly a matter of concern not only for the above estuaries where harbours are implanted but also for the adjacent continental shelves, regardless the massive dilution of contaminants that may occur in these deeper areas.

Key words: *Nassarius reticulatus, Imposex, Butyltins, Phenyltins, Octyltins, Inshore/offshore gradients*

3.1 INTRODUCTION

In the beginning of the 1970's some authors reported a very sudden and odd upsurge of gastropod females with male characters along the Atlantic coasts of Europe and USA (Blaber, 1970; Houston, 1971; Poli *et al.*, 1971; Smith, 1971). Smith (1971) designated this phenomenon by imposex, the superimposition of male characters (notably a penis and a vas deferens) onto females of gonochoristic gastropods. Nowadays, imposex is a common and worldwide distributed phenomenon affecting many prosobranch species. To our best knowledge, imposex is specifically caused by tributyltin (TBT) and, in some species, triphenyltin (TPT) pollution. This pollution is globally spread due to the large use of TBT and, to a lesser extent, TPT, as biocides in ships' antifouling paints since the mid 1960s. Many studies also proved that these compounds were toxic to a broad taxonomic range of organisms, from bacteria to vertebrates, causing a variety of negative impacts on the marine ecosystems (Alzieu, 2000; Mendo *et al.*, 2003; Shimasaki *et al.*, 2003; Kungolos *et al.*, 2004). Considering the high environmental risks of these compounds, an International Convention on the Control of Harmful Anti-Fouling Systems on Ships (AFS-Convention) was adopted on 5 October 2001 at a Diplomatic Conference held under the aegis of the International Maritime Organization (IMO). As an immediate follow-up to the AFS-Convention, the European Union (EU) adopted the Directive 2002/62/EC and, later on, the Regulation 782/2003 for the prohibition of the application of organotin compounds on ships flying the flag of a Member State (irrespective of their length) from 1 July 2003 and for the elimination of the presence of organotin compounds on ships after 1 January 2008. Moreover, fentin acetate and fentin hydroxide, the agrochemical TPT fungicides, received non inclusion decisions under directive 91/414/EEC in 2002 (2002/478/EC and 2002/479/EC). European Member States had to remove all products containing these active substances from the market by December 2002 and farmers had to have used all stocks by December 2003. However, this pollution is still under great concern as ships could legally carry these organotin paints till 2008 and inputs may still occur for many years due to TBT remobilization from past contaminated sediments (de Mora *et al.*, 1995).

Imposex is undoubtedly the most reliable and currently used biomarker for monitoring TBT pollution and, consequently, intensive imposex monitoring surveys have been carried out along extensive coastal areas around the world. However, these surveys are generally confined to the innermost coastal waters such as estuaries, lagoons and bays that enclose ports, marinas and dockyards. Remarkably, there is still little information regarding the inshore/offshore organotin distribution and the associated impacts on deeper continental shelves. Because of the massive dilution of contaminants that occurs in these areas the methodological challenge of these kind of works concerns the achievement of very low chemical detection limits for the compounds under study (and their degradation products) in combination with representative samples of bioindicator organisms and the use of very sensitive and specific biomarkers of effects.

Rato *et al.* (2006) assessed the imposex and organotin contamination of the netted dogwhelk *Nassarius reticulatus* (L.) in north Portugal between 2002 and 2005 over an area of 735 km^2, in order to evaluate the dispersion of organotins from inshore sources into the adjacent deeper sea. These authors found that offshore populations were extensively affected by imposex and contaminated by TBT (the dominant organotin) and concluded that the impact of this pollution around the world may be much worse that initially thought considering the huge spatial extension involved. These results prompted the need to extend the survey to the central and southern coasts of Portugal using the same indicator species.

Nassarius reticulatus is a ubiquitous prosobranch in the European coast (Graham, 1988) that was proposed as a bioindicator of TBT pollution by Stroben *et al.* (1992b) and recommended as a suitable TBT indicator species by OSPAR (2003a). Rato *et al.* (2006) showed that *N. reticulatus* is very common at depths of at least 34 m off north Portugal, so it is potentially a good candidate for biomonitoring TBT pollution further south on the continental shelf. Hence, the objective of the present work is to combine measurements of imposex and organotin body burden in *N. reticulatus* populations in the continental shelves around the main estuaries/lagoons of the central (Lisbon and Setúbal) and southern (Faro) Portuguese coasts, in order to evaluate the magnitude and effects of organotin pollution in these areas and characterize the inshore/offshore gradients of organotin compounds.

3.2 MATERIAL AND METHODS

3.2.1 Study area

The study area (Figure 3.1) is divided in three regions: Lisbon (between 38º 29.00N and 38º 39.00N), Setúbal (between 38º 20.00N and 38º 28.00N) and Faro (between 07º 47.50 W and 07º 57.50 W). The Lisbon region includes the Tagus estuary, which is the largest estuary in Portugal and one of the largest on the European Atlantic coast, with 50 km in length and an area of 325 km^2, of which about 40% is intertidal area (França *et al.*, 2005) and has a single entrance to the sea. The Setúbal region includes the Sado estuary, the second largest in Portugal, with an area of approximately 240 km^2, which comprises a wide bay and a narrow channel through which Sado River enters into the system (Caeiro *et al.*, 2005). The Sado estuary has also a single entrance to the sea. The Faro region comprises the Ria Formosa, a shallow coastal lagoon, of approximately 100 km^2 with reduced freshwater inputs and intensive water mass exchange with the sea through more than one entrance (Serpa *et al.*, 2007). It is a complex system of salt marshes and tidal flats, separated from the Atlantic by a belt of sand dunes that extend for 55 km along the coast (Aníbal *et al.*, 2007).

The Tagus and Sado estuaries and the Ria Formosa may represent important sources of organotin pollution since they harbour commercial and fishing ports, marinas and shipyard facilities, which in most cases are close to each other. Table 3.1 summarises relevant information regarding boat traffic and shipyard activity in each estuary: Lisbon is the most important port (in fact the main port in the country) followed respectively by Setúbal and Faro; the same rank is observed for fishing/leisure boat traffic and dockyard activity.

3.2.2 Sampling

Nassarius reticulatus was sampled between June and October 2006 in the regions of Lisbon, Setúbal and Faro. The Lisbon survey, covering approximately 100 km^2, consisted of 2 sampling sites located inside the Tagus estuary and 43 sampling sites distributed along 11 transects in the offshore region.

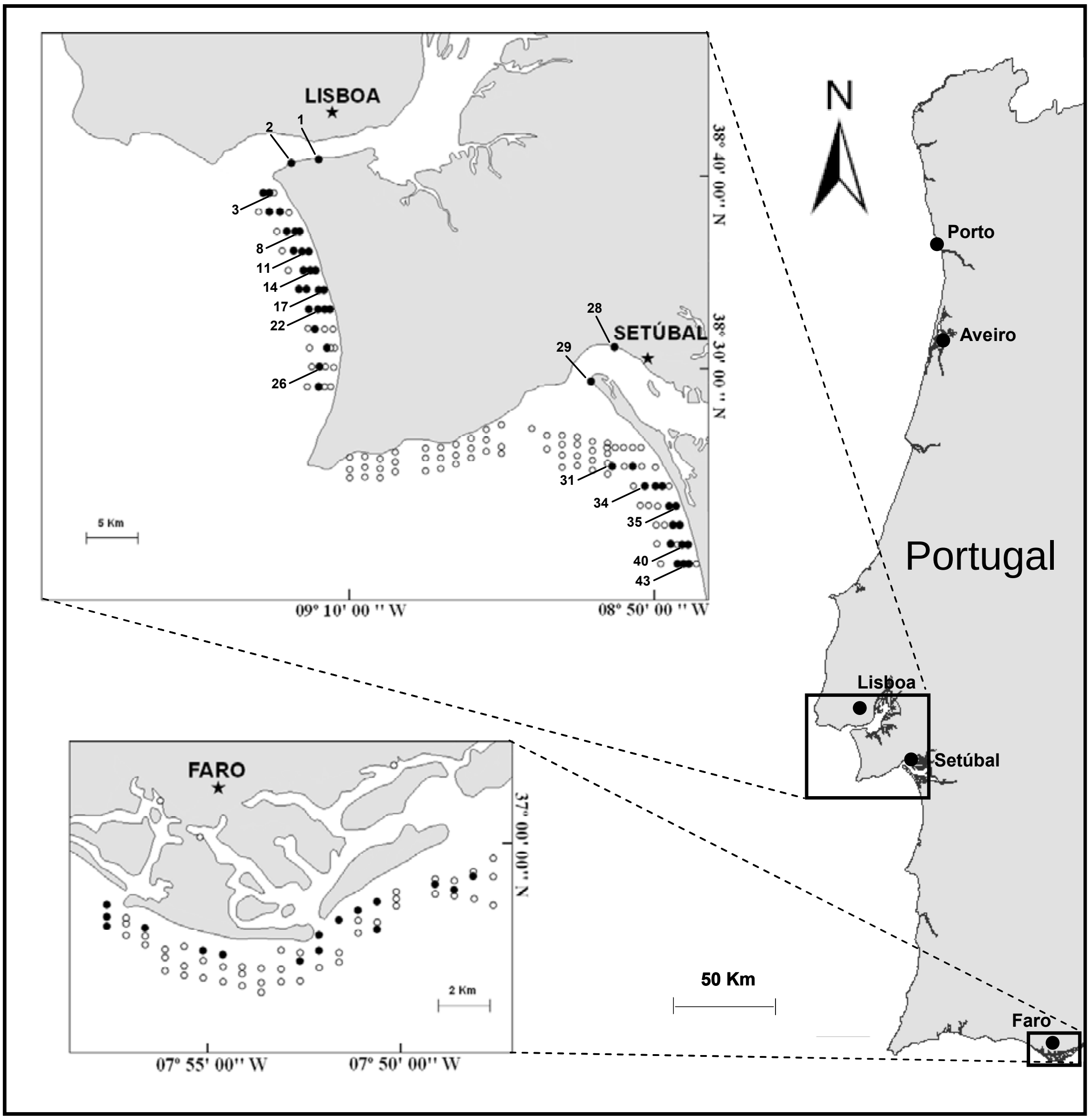

Figure 3.1 – Map showing the study area and location of the sampling sites. Black circles represent sites where samples with 4 or more females were obtained and white circles represent sites where no females or a very low number of females (<4) were obtained and so were not used in the analysis. The numbers correspond to sites where samples were also analyzed for organotin content (compare Table 3.3).

The Setúbal survey, around 125 km^2, included 2 sampling sites inside the Sado estuary plus 83 sampling sites distributed along 23 transects in the offshore region. The Faro survey, which enclosed approximately 25 km^2, consisted of 60 offshore sampling sites distributed along 20 transects plus 3 other sites located inside the Ria Formosa.

Transects were perpendicular to the coast and were separated approximately 1 mile from each other in Lisbon and Setúbal regions, and approximately 0.5 miles in Faro region. On each transect, 1-5 sampling stations were distributed between the bathymetric lines of 3 and 20 m (Figure 3.1). The positioning of these sites was performed with a global positioning system and mapped using the ArcGis software from ESRI (Redland, CA, USA). Samples were collected on board the RV Diplodus and consisted of a 5 minute tow using 2 dredges, one on each side of the boat. Each dredge was 0.64 m in width and carried a net bag of 35 mm mesh size. The total area surveyed at each site was approximately 140 m^2.

Table 3.1 – Characterization of boat traffic and shipyard activity inside the Tagus estuary (Lisbon), Sado estuary (Setúbal) and Ria Formosa (Faro) that are comprised in the study area, with indication of: type of port (Main, Secondary), number of commercial ships entered in 2003, 2004 and 2005 and respective tonnage (GT – Gross Tonnage), and number of marinas, dockyards and fishing ports.

		Nº of ships entered[a]			Tonnage (GT)[a]			Nº of	Nº of	Nº of fishing
Port	**Type[a]**	**2003**	**2004**	**2005**	**2003**	**2004**	**2005**	**marinas**	**dockyards**	**harbours**
Lisbon	M	3.522	3.270	3.351	40.219.138	35.952.814	38.568.904	6[b]	18[b]	2[e]
Setúbal	M	1.617	1.666	1.608	16.715.224	17.309.881	16.923.056	3[c]	1[c]	2[e]
Faro	S	49	33	38	141.428	90.474	96.111	2[d]	1[d]	1[e]

a - Source: National Statistical Institut - Port Administrations; M - main port; S - small port
b - Source: APL - Port of Lisbon Administration
c - Source: APSS,SA - Port of Setúbal and Sesimbra Administration
d - Source: IPTM - Port and Marine Transports Institut
e - Source: Docapesca Portos e Lotas, SA

3.2.3 Imposex analysis

N. reticulatus specimens were maintained alive in aquaria with artificial sea water (35 psu) and examined for imposex within 3 days after the collection. For imposex analysis only adult animals were used (i.e. those with white columellar callus and teeth on the outer lip) and were randomly selected from each sample. Only samples consisting of four or more females were used. The shell height (distance from shell apex to lip of siphonal canal) was measured with Vernier calipers to the nearest 0.1 mm. For the estuarine samples we selected 15 females that presented shell height values around the average observed in the offshore area. After about 40 minutes of narcotization using 7% $MgCl_2$ in distilled water, shells of *N. reticulatus* were cracked open with a bench vice and individuals were sexed and dissected under a stereomicroscope. Parasitized specimens were discarded. The relative penis length index (RPLI = mean female penis length × 100/mean male penis length), the mean female penis length index (FPLI), the vas deferens sequence index (VDSI) and the percentage of affected females (%I) were determined for each station. The VDS was classified according to the scoring system proposed by Stroben *et al.* (1992b), except that stage 4+ was converted to stage 5 for computation of mean values at each site. The penis length was measured using a stereomicroscope with a graduated eyepiece to the nearest 0.14 mm. Among all the imposex indices the VDSI is the most meaningful biological parameter since it provides a direct indication of the average reproductive capacity of the females in the population. Besides, many females in this study presented b-type VDS stages, i.e. with no penis development (Stroben *et al.*, 1992b), which reduces the usefulness of penis length based indices such as FPLI and RPLI. For this reason VDSI is the index selected to represent imposex for correlation analysis. Throughout this paper correlation analysis refers to the Pearson product-moment coefficient after checking for normality of the data through the Kolmogorov-Smirnov test.

3.2.4 Organotin analysis

Sub-samples of about 10 *Nassarius reticulatus* females were used for the quantification of organotin concentration in the tissues. These samples were randomly selected along transects from the estuaries to the outer sea in the Lisbon and Setúbal regions in order to assess inshore/offshore contamination gradients. The analysis of organotin compounds, mono- to trisubstituted butyltins (MBT, DBT, TBT), phenyltins (MPT, DPT, TPT) and octyltins (MOcT, DOcT, TOcT), were performed following the method described by Iwamura *et al.* (2000) with some modifications. Approximately 1 g of freeze-dried gastropod tissue was spiked with 50 ng of internal standards including deuterated butyltins (d9-MBT, d18-DBT and d27-TBT), phenyltins (d5-MPT, d10-DPT and d15-TPT), and octyltins (d17-MOcT, d34-DOcT and d51-TOcT) (50µL of solution containing 9 deuterated organotins dissolved by ethyl acetate at each concentration of 1 µg/mL) and homogenized with 35mL of 1N HBr/methanol-ethyl acetate (1:1) solution by Polytron homogenizer.

The homogenate sample was centrifuged (15 min at 3000 rpm), and the supernatant was transferred to a decantation balloon with 50ml of NaBr saturated water and 15ml of ethylacetate/hexane (3:2). Again, 35mL of 1N HBr/methanol-ethyl acetate (1:1) solution was added into the centrifuged sample (residue), homogenized and centrifuged (15 min at 3000 rpm). This supernatant was also transferred to decantation balloon. After extraction (by shaking for 10 min, twice) 100 ml of hexane was added into the extract and water phase was discarded. Then the organic layer was dehydrated with anhydrous sodium sulphate and concentrated using a rotary evaporator near to dryness. The concentrate was solved into 5 mL of ethanol, 10 mL of ultra pure water and mixed with 5 ml of 1M acetate buffer (pH 5.0). Organotins in the extract were then ethylated by adding 1 ml of 5% tetraethyl sodium borate. After ethylation (by shaking for 15 min) 40 ml of 1M KOH was added to the mixture that was shaken one hour to decompose the fat. After saponification the ethylated organotins were re-extracted by 40 mL of hexane (by shaking for 10 min, twice). Afterwards the hexane extract was dehydrated by sodium sulphate, concentrated by a rotary evaporator near to dryness. The concentrate was added into a SEP-PAK plus florisil cartridge, and organotins were eluted by 8 mL of 5% diethylether/hexane. The final solution was concentrated under a gentle nitrogen flux to 1 ml and spiked with 50 ng of deuterated tetrabutyltin (50μL of solution dissolved by hexane at concentration of 1 μg/mL) as a recovery standard. The final solution was then injected into a gas chromatograph.

The quantification of organotin compounds was conducted by a gas chromatograph equipped with a mass spectrometer (GC-MS) (Hewlett-Packard 6870 GC system with 5973 mass selective detector and 7683 series auto sampler). GC-MS was equipped with a fused silica capillary column [0.25 mm i.d. x 30m length consisted of DB-1 (100% dimethylpolysiloxane, 0.25 μm bounded phase)] and operated in electron impact and selected ion monitoring mode (EI-SIM). The concentrations of organotin compounds were calculated based on the peak areas of target compounds and their deuterated surrogates as internal standards following an internal standard isotope dilution method. Calibration curves for MBT, DBT, TBT, MPT, DPT, TPT, MOcT, DOcT, TOcT were made from the analysis of standard solutions showing 4 levels of native compound concentrations (5, 10, 50 and 250 ng/ml) with a constant concentration of internal and recovery standards (50 ng/ml). Recoveries of internal standards through the whole analytical procedure were estimated based on the peak areas of internal and recovery standards. Except for MPT and MBT, the recoveries of internal standards were within 60%~100%: average recovery rates (±St Dev) of MBT, DBT, TBT, MPT, DPT, TPT, MOT, DOT and TOT were 48.9±5.21, 84.8±11.95, 80.1±7.03; 29.4±14.39, 67.1±11.51, 107.8±13.19; 61.1±15.48, 82.3±12.01 and 97.6±11.71, respectively. We did not estimate concentrations of MPT in this study because of low recoveries of an internal standard for MPT. Recoveries of internal standards for MBT were around 50 %, meaning that the concentrations of this compound must be considered as reference values.

To assess the QA/QC of measurements in this study, certified reference material of fish tissue (NIES CRM No.11) was analyzed by the method described above. The CRM No.11 has the certified concentration value for TBT at 1.3±0.1μg/g (concentration is dry weight basis as chloride foam), and the (non-certified) reference value for TPT at 6.3μg/g. Our data obtained from the analysis of this CRM showed a good agreement: TBT concentrations in CRM No.11 (n=3) was 1.2±0.03μg/g and TPT concentration (n=3) was 6.3±0.04μg/g. In addition, a procedural blank was included with each analytical batch to check for interfering compounds and to correct sample values, if necessary. The detection limits of each organotin compound were calculated based on deviation (3σ) of each peak area when the standard solutions containing low levels of native compounds (1 or 5 ng/ml) were measured by GC-MS. If any peak was detected in the blank sample, detection limit was determined as quantities of three times those peak areas. In this study, concentrations of organotin compounds were described in terms of ng Sn/g dry wt.

3.3 RESULTS

3.3.1 Imposex

A total of 1712 specimens were analyzed, of which 55% were females and 45% were males. The abundance of *N. reticulatus* seems to increase with the latitude because a relatively small number of animals were obtained in Faro but this number increased progressively in direction to Lisbon. Among the 193 sampled sites only 60 sites provided samples with 4 or more females and only these samples were in fact used for the current work (Table 3.2; Figures 3.2, 3.3). No sterilized females were found in the present study.

Table 3.2 – *Nassarius reticulatus*. Data relative to samples collected along the study area with indication of the location (N – North; W- West), region (L – Lisbon, S – Setúbal, F – Faro), distance (length, in kilometres, between the sampling station and the inner station in the estuary), depth (in meters), the vas deferens sequence index (VDSI), the mean female penis length index (FPLI), the relative penis length index (RPLI = mean female penis length*100/mean male penis length), the number of males (N ♂), and the number of females (N♀). Blank cells in RPLI column correspond to samples with less than 4 males (see text). Sampling sites in Lisbon and Setúbal are ordered according to their location from North to South and, for each transect, from East to West; in Faro they are ordered from East to West and, for each transect, from North to South.

	Coordinates			Distance	Depth					
Code	**N**	**W**	**Region**	**(km)**	**(m)**	**VDSI**	**FPLI**	**RPLI**	**Nº ♂**	**Nº ♀**
1	38º 40.77	09º 12.29	L	0.00	4	3.33	2.92	22	15	15
2	38º 40.55	09º 14.09	L	2.2	2	3.53	3.95	36	15	15
3	38º 39.00	09º 15.53	L	10.17	7	1.42	0.37	3	2	6
4		09º 15.91	L	10.16	9	1.67	1.20	9	7	19
5	38º 38.00	09º 14.80	L	12.14	8	1.17	1.07	10	10	6
6		09º 15.48	L	12.04	11	1.40	0.38		3	10
7	38º 37.00	09º 13.47	L	14.55	5	1.97	0.45	4	27	33
8		09º 13.78	L	14.21	7	1.74	0.40	2	21	39
9		09º 14.31	L	13.99	10	1.32	0.20	2	21	38
10	38º 36.00	09º 12.86	L	16.61	6	1.29	0.07	1	26	14
11		09º 13.30	L	16.44	9	1.17	0.16	1	30	18
12		09º 13.88	L	16.25	12	1.17	0.21	2	15	29
13	38º 35.00	09º 12.38	L	18.59	4	0.89	0.05	1	16	18
14		09º 12.77	L	18.39	6	0.87	0.03	0	29	31
15		09º 13.22	L	18.27	9	0.90	0.16	1	21	39
16	38º 34.00	09º 11.85	L	20.58	5	1.04	0.80	1	33	27
17		09º 12.16	L	20.39	8	0.93	0.26	2	42	30
18		09º 12.98	L	20.18	11	1.09	0.53	3	15	45
19		09º 13.48	L	20.04	16	0.25	0.00	0	6	4
20	38º 33.00	09º 11.43	L	22.54	4	0.20	0.00	0	17	10
21		09º 11.78	L	22.39	7	0.91	0.11	1	28	32
22		09º 12.19	L	22.21	10	0.94	0.18	1	26	34
23		09º 12.79	L	22.04	15	1.20	0.32		3	6
24	38º 32.00	09º 12.38	L	23.9	20	0.31	0.00	0	6	13
25	38º 31.00	09º 11.57	L	25.87	12	0.08	0.00	0	12	12
26	38º 30.00	09º 12.08	L	27.53	14	0.13	0.00	0	8	8
27	38º 29.00	09º 12.10	L	29.38	14	0.03	0.04	0	9	11
28	38º 31.17	08º 52.58	S	0.00	3	4.13	5.87	33	15	15
29	38º 29.40	08º 54.14	S	3.21	3	4.07	4.53	64	15	15
30	38º 25.00	08º 51.33	S	15.21	12	0.17	0.00	0	9	6
31		08º 52.67	S	14.62	20	0.09	0.00	0	13	47
32	38º 24.00	08º 49.39	S	17.69	6	0.08	0.00		3	13
33		08º 49.84	S	17.47	9	0.08	0.00	0	7	14
34		08º 50.55	S	17.16	12	0.22	0.00	0	22	36
35	38º 23.00	08º 48.79	S	19.84	3	0.14	0.00	0	8	21
36		08º 48.90	S	19.52	9	0.38	0.00	0	7	8
37	38º 22.00	08º 48.19	S	21.93	5	0.00	0.00	0	4	4
38		08º 48.67	S	21.69	7	0.00	0.00	0	12	10
39	38º 21.00	08º47.64	S	23.61	3	0.18	0.00	0	14	22
40		08º 48.03	S	23.31	7	0.33	0.21	0	17	27
41		08º48.82	S	23.03	15	0.20	0.00		3	5
42	38º 20.00	08º 47.59	S	25.75	5	0.00	0.00		3	4
43		08º 47.94	S	25.43	7	0.17	0.00	0	6	6
44		08º 48.33	S	25.16	12	0.28	0.00	0	10	18
45	36º 59.33	07º 48.00	F		10	0.00	0.00	0	12	7
46	36º 59.05	07º 48.50	F		8	0.00	0.00		2	4
47	36º 59.16	07º 49.00	F		5	0.00	0.00	0	5	4
48	36º 58.80	07º 50.50	F		3	0.33	0.04	0	8	9
49	36º 58.72		F		8	0.00	0.00	0	7	4
50	36º 58.63	07º 51.00	F		3	0.00	0.00	0	6	6
51	36º 58.43	07º 51.50	F		8	0.27	0.00	0	6	11
52	36º 58.13	07º 52.00	F		3	0.00	0.00		3	4
53	36º 57.78		F		8	0.00	0.00		2	4
54	36º 57.57	07º 52.50	F		7	0.00	0.00		3	4
55	36º 57.72	07º 54.50	F		3	0.00	0.00	0	7	9
56	36º 57.80	07º 55.00	F		8	0.00	0.00	0	4	4
57	36º 58.28	07º 56.50	F		8	0.14	0.00		3	7
58	36º 58.75	07º 57.50	F		3	0.17	0.00	0	5	6
59	36º 58.51		F		5	0.00	0.00	0	7	5
60	36º 58.31		F		10	0.17	0.00	0	9	6

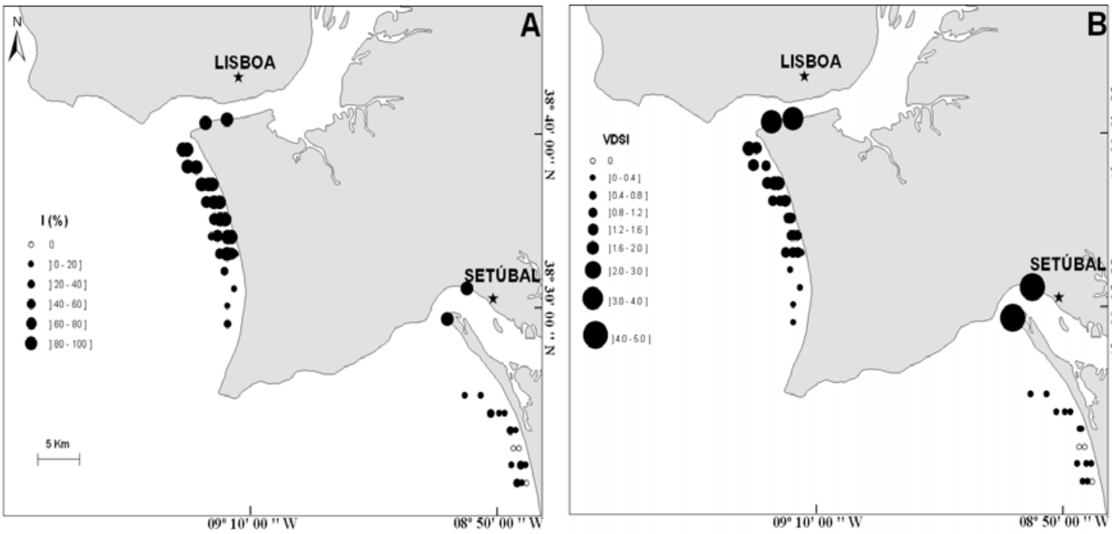

Figure 3.2 – *Nassarius reticulatus*. Map of the Portuguese continental shelf between Lisbon and Setúbal indicating the spatial distribution of (**A**) imposex incidence (%I) and (**B**) vas deferens sequence index (VDSI).

In Lisbon females affected with imposex occurred in all samples but the prevalence and severity of imposex decreased clearly with the distance from the Tagus estuary (Figure 3.2). In fact, a highly significant negative correlation was observed between the imposex intensity (VDSI) and the distance from the Tagus estuary ($r=-0.91$, $P<0.001$) (Figure 3.4a). Across the sites the percentage of females with imposex (%I) varied between 8 and 100%, the VDSI ranged between 0.03 and 3.53, the FPLI varied between 0.00 and 3.95mm and the RPLI spanned from 0 to 36%; all these parameters decreased with the distance from the estuary (Table 3.2; Figure 3.2).

In Setúbal we found females with imposex in 14 of the 17 sites with available samples. Across sites the %I varied between 0 and 100%, the VDSI ranged between 0.00 and 4.13, the FPLI varied from 0.00 to 5.87 mm and the RPLI ranged between 0% and 64% (Table 3.2; Figure 3.2). The imposex levels in the offshore-side area were also lower than in the Sado estuary. In this case, the correlation between imposex levels and distance from Sado estuary was not applied since there are no values between a group of two points with a VDSI of approximately 4 (corresponding to sites inside the estuary) and the remaining points (outside the estuary) presenting a VDSI close to 0 (Figure 3.4c); the gap in the middle of these groups happened because no animals occurred in the samples between 0 and 10 km from the mouth of the estuary.

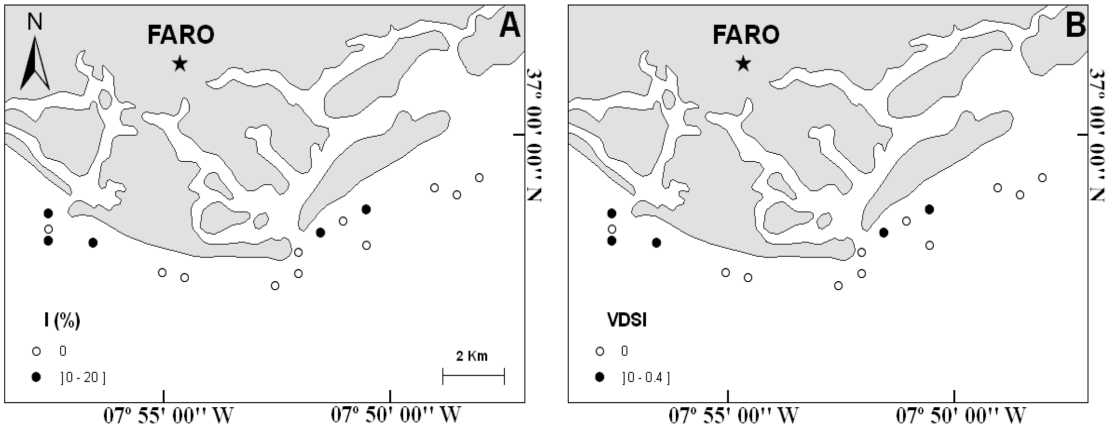

Figure 3.3 – *Nassarius reticulatus*. Map of the continental shelf adjacent to Faro indicating the spatial distribution of (**A**) imposex incidence (%I) and (**B**) vas deferens sequence index (VDSI).

In Faro no specimens were obtained inside the Ria Formosa. The %I varied from 0 to 17%, the VDSI ranged between 0.00 and 0.33, the FPLI varied between 0.00 and 0.04 and the RPLI was always close to 0 (Table 3.2, Figure 3.3). It is difficult to characterize adequately the spatial pattern of imposex in this region due to the low number of animals sampled but a higher degree of imposex occurred near the entrances of Ria Formosa.

3.3.2 Organotins

In the regions of Lisbon and Setúbal the butyltins (BTs) were always above the detection limits and represented, respectively, an average of 90% and 98% of the total organic tin (ΣOT) across sites, which constitutes by far the highest fraction of organotin compounds quantifiable by our method. In the region of Lisbon the TBT concentration represented an average of 33% of total BTs and varied between 8.4 and 99.0 ng Sn/g dry wt. In Setúbal the TBT concentration represented an average of 44% of total BTs and varied between 3.6 and 120.0 ng Sn/g dry wt (Table 3.3). A significant negative correlation occurs between the Ln TBT concentration in the tissues of *N. reticulatus* and the distance from estuaries in Lisbon ($r=-0.81$, $P<0.01$) and Setúbal ($r=-0.97$, $P<0.001$) (Figure 3.4b and 3.4d), which clearly indicates the existence of an inshore/offshore decreasing gradient of

TBT pollution and imposex (Figure 3.4e) from the estuaries to the sea. DBT varied between 8.2 and 59 ng Sn/g dry wt in Lisbon and between 3 and 39 ng Sn/g dry wt in Setúbal, representing, respectively, an average of 25% and 30% of total BTs.

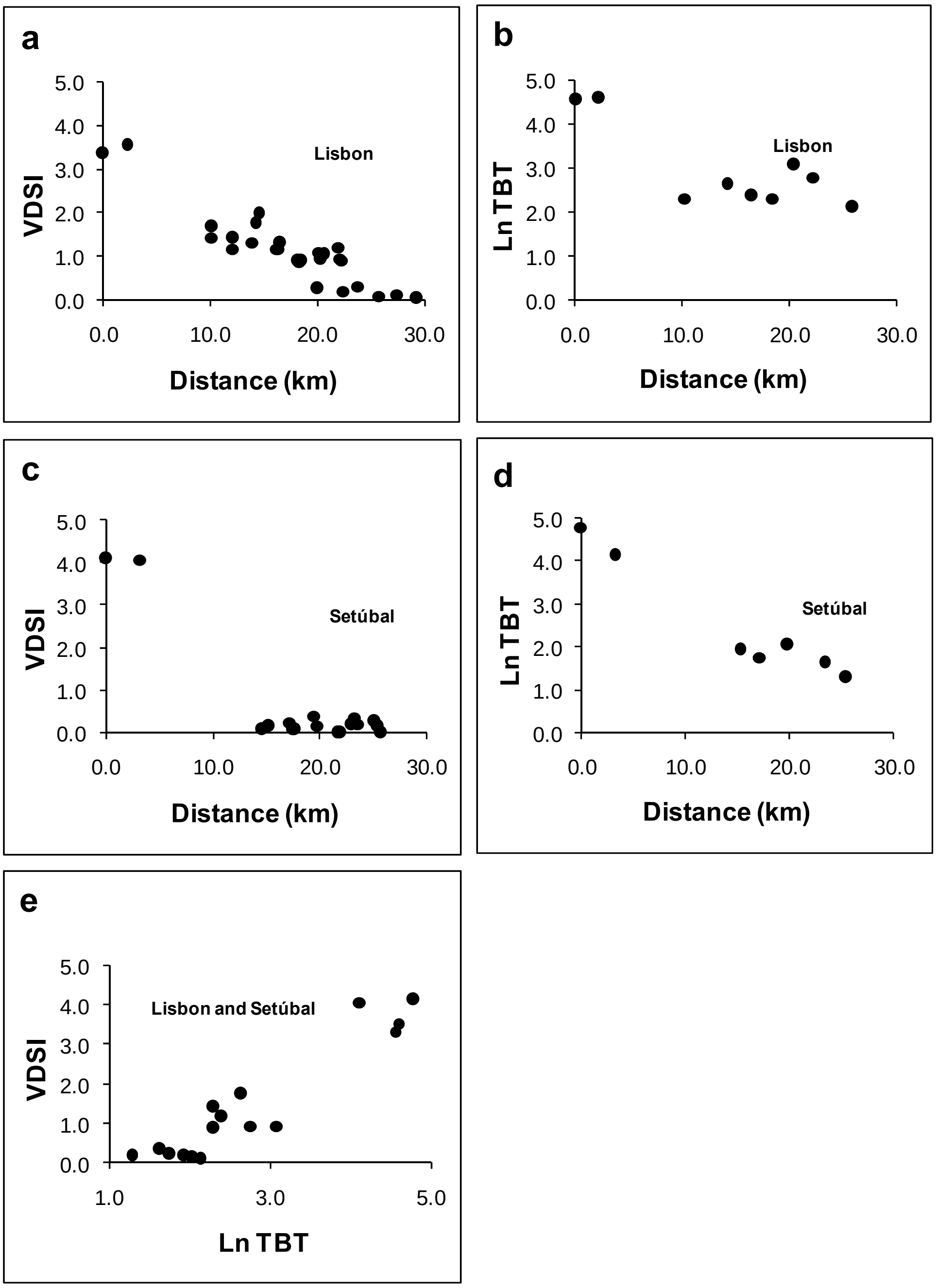

Figure 3.4 – *Nassarius reticulatus*. Correlation analysis between the imposex (VDSI) and the distance from ports in Lisbon (**a**) and Setúbal (**c**); correlation between the tributyltin (TBT) tissue concentration and distance from ports in Lisbon (**b**) and Setúbal (**d**); correlation between VDSI and TBT tissue concentration (**e**).

MBT ranged from 13 to 97 ng Sn/g dry wt in Lisbon and from <3.2 up to 97 ng Sn/g dry wt in Setúbal, representing 42% and 26% of total BTs. Highly significant positive correlations between TBT and DBT ($r=0.94$, $p<0.001$) and between TBT and MBT ($r=0.93$, $p<0.001$) were observed. The phenyltin compounds represented only 10% and 2% of ΣOT in Lisbon and Setúbal, respectively; triphenyltin was the dominant phenyltin (84% and 70%, respectively) with values of up to 6.8 ng Sn/g dry wt in Lisbon and up to 4.9 ng Sn/g dry wt in Setúbal (Table 3.3). No significant correlation was observed between TPT and the distance from the estuaries in both regions. The octyltin compounds were always below the detection limit and are thus negligible (Table 3.3). No organotin analyses were performed for the region of Faro.

Table 3.3 – *Nassarius reticulatus*. Monobutyltin (MBT), dibutyltin (DBT), tributyltin (TBT), monophenyltin (MPT), diphenyltin (DPT) and triphenyltin (TPT) concentrations (ng Sn/g dry wt) in the whole tissues of females across sampling stations. All samples were below the detection limits for monoctyltin (MOcT), dioctyltin (DOcT) and trioctyltin (TOcT).

Station code	MBT	DBT	TBT	DPT	TPT
1	55	43	95	<0.2	<0.2
2	97	59	99	0.9	5.2
3	16	9	10	<0.2	5.5
8	18	12	14	1.7	5.8
11	25	11	11	1.8	5.8
14	18	8.2	10	1.3	4.6
17	27	16	22	1.1	6.8
22	21	14	16	0.8	4.3
26	13	9	8.4	0.4	2.6
28	97	39	120	1.2	4.9
29	88	39	62	2.2	<0.2
31	5.5	4.7	6.9	<0.2	<0.2
34	5.5	3.8	5.8	<0.2	0.7
35	4.6	5.5	7.7	<0.2	0.8
40	<3.2	4.3	5.1	<0.2	<0.2
43	<3.2	3	3.6	<0.2	<0.2

Detection limits: MBT - 3.2 ng Sn/g; DBT and TBT - 0.5 ng Sn/g; DPT and TPT - 0.2 ng Sn/g; MOcT – 2.88 ng Sn/g; DOcT - 2.6 ng Sn/g; TOcT - 1.0 ng Sn/g

3.4 DISCUSSION

The current study shows that imposex in *Nassarius reticulatus* is widely spread throughout the continental shelves around the main estuaries of the central and south coast of Portugal. In Lisbon and Setúbal imposex showed a decreasing gradient from the estuaries to the offshore. The present study also shows the occurrence of a highly significant correlation ($r=0.94$, $P<0.001$) between the level of the TBT contamination of *N. reticulatus* tissues and the intensity of imposex (VDSI) across stations in Lisbon and Setúbal (Figure 3.4e). Similar correlations have been also reported for this species in other areas of the coast of Portugal (Barroso *et al.*, 2000, Barroso and Moreira, 2002; Barroso *et al.*, 2002a; Rato *et al.*, 2006), Spain (Barreiro *et al.*, 2001), France (Stroben *et al.*, 1992a) and Britain (Bryan *et al.*, 1993). These field correlations provide further evidence of the cause-effect relationship established from laboratory experiments: imposex in *N. reticulatus* is caused by TBT, whether administered by injection, by aqueous and sediment exposure or through the diet (Stroben *et al.*, 1992b; Bettin *et al.*, 1997; Pope, 1998; Barroso *et al.*, 2002b) . The present work also shows that the levels of TPT residues in the tissues are much lower than TBT but nevertheless they represent an average of 32% of the total amount of TBT in Lisbon, though only 4% in Setúbal. As imposex is suggested to be triggered in the laboratory at higher levels of TPT in comparison to TBT (Barroso *et al.*, 2002b), imposex in these populations is probably attributed mainly to TBT pollution.

The major usage of TBT is as an antifouling agent in boat paints (Bennet, 1996). In aquatic environments TBT is released from TBT-antifouling coatings of the ship/boat hulls and from dockyard hydroblasting wastes and so the ports, marinas and dockyards are considered to be the main sources of TBT pollution (Evans *et al.*, 1995; Barroso *et al.*, 2002a; Bech, 2002; Gibson and Wilson, 2003; Harino *et al.*, 2005). The significant negative correlation found between the Ln TBT concentration in the tissues of *N. reticulatus* and the distance from estuaries in the Lisbon and Setúbal regions is indicative that the estuaries may be in fact the point sources of pollution in those areas, since they embrace important naval facilities. The main TBT inputs to adjacent offshore areas may originate from water currents flowing from estuaries. Besides, the navigational lanes that run from the sea to the mouth of the estuaries may also constitute a source of pollution for the involving regions. Additionally, immersion of dredged estuarine sediments into nearby offshore areas may also constitute a source of TBT (see Santos *et al.*, 2004) but as they are deposited far away from the surveyed areas their contribution is probably of minor importance. It is evident that boat traffic and shipyard activity is much higher in Tagus and Sado estuaries than in Ria Formosa (Table 3.1) and consequently imposex is more intense in the former regions (Figures 3.2 and 3.3).

MBT and DBT are used in many industrial applications, for instance, the former is commonly used as stabiliser for PVC and glass coating whereas DBT is generally used as PVC stabiliser and as catalyst agent (Bennet, 1996; WHO, 2006). Nevertheless, the highly significant positive correlation found in the current study between the concentrations of TBT and these compounds in the tissues (respectively, $r=0.93$, $P<0.001$ and $r=0.94$, $P<0.001$) suggests that their main source can be the degradation from TBT. In fact, the significant negative correlation found between the proportion of TBT relative to the total BTs and the distance from the Tagus estuary ($r=0.73$, $P<0.05$) may suggest that a progressive degradation of this compound in the marine water, with a typical half-life of 1-3 weeks (Hoch, 2001), may occur from the point sources inside the estuary to the offshore. However, no significant correlation was observed between the proportion of TBT and the distance from the Sado estuary, probably related to the lack of data for the correlation analysis regarding animals between 0 and 10 km outside the estuary.

TPT has been used as co-biocide in antifouling paints – at much lower concentrations than TBT – and was used as a pesticide in agriculture (Fent, 1990). In the present study we did not find a significant correlation between TBT and TPT in the tissues and so it is difficult to gather the real source of this compound. The octyltin compounds which are mainly used as stabilizers in PVC (Hoch, 2001) were always below the detection limit and are thus negligible for the current study.

The current work shows that TBT pollution is undoubtedly a matter of concern in the Portuguese continental shelf, particularly in Lisbon, where imposex in *N. reticulatus* is extensively spread due to this pollutant. The elevated pollution levels inside the estuaries certainly cause adverse ecological effects (Barreiro *et al.*, 2001; Barroso *et al.*, 2002a; Barroso *et al.*, 2004) and great economical losses - for instance, the oyster *Crassostrea* sp. production in the Tagus and Sado estuaries is presumed to have collapsed in the mid 1970s due to the upsurge of organotin pollution (Dias, 1990). This is of great concern but the most relevant aspect shown in this study refers to the magnitude of the spatial extension of TBT pollution outside the estuaries. In fact, females with imposex were found is almost all samples (93%) obtained in the Lisbon and Setúbal offshore-side areas. Besides, in deeper areas of the Lisbon continental shelf more than half the sites registered a VDSI between 1 and 2, which in this species is indicative of a TBT contamination in water approximately in the range of 1 to 3.5 ng TBT-Sn/L (Huet *et al.*, 1995). The no-observed-adverse-effect (NOEC) level proposed by Alzieu and Michel (1998) implies that planktonic organisms and molluscs are likely to be the most affected; suggested NOEC levels are 0.5 ng TBT-Sn/L for imposex in gastropods, 0.5 ng TBT-Sn/L for phytoplankton and zooplankton growth and 0.7 ng TBT-Sn/L for calcification anomalies in *Crassostrea gigas*.

TBT has been selected by OSPAR (Convention for the Protection of the Marine Environment of the North-East Atlantic) as a chemical for priority action and is also considered a priority hazardous substance according to the EU Water Framework Directive (European Parliament, 2000). Monitoring TBT pollution in the aquatic environment is mandatory of the OSPAR Coordinated Environmental Monitoring Programme (CEMP) (OSPAR, 2004) which has developed assessment criteria for monitoring TBT-specific biological effects. These criteria include imposex as a TBT-specific biomarker for evaluating the risk of TBT contamination to marine ecosystems. For simplicity, we analyse here if VDSI values fall into the OSPAR assessment classes that are above or below the critical point of 0.3. According to the assessment criteria for *N. reticulatus*, a VDSI higher than 0.3 indicates exposure to TBT concentrations above the Environmental Assessment Criteria (EAC) derived for TBT in water, i.e., there is at least a risk of adverse effects such as reduced growth and recruitment, in the more sensitive taxa of the ecosystem caused by long-term exposure to TBT. Hence, the results of this work show that, in the Lisbon region, 21 of the 25 offshore-side stations (VDSI ranging between 0.31 and 1.97) were exposed to TBT concentrations higher than the EAC. The offshore-side stations in Setúbal cannot be compared to Lisbon as animals could not be collected in the proximity of the estuary; even so, 2 of the 15 stations (Stns. 36 and 40; VDSI, respectively, 0.38 and 0.33) in Setúbal were slightly above the EAC derived for TBT. The stations inside the Tagus estuary (VDSI of 3.33 and 3.47) and the Setúbal estuary (VDSI of 3.87 for both stations) (VDSI computed according to Stroben *et al.*, 1992a) were exposed to TBT concentrations far above the EAC value; the high VDSI scores registered may indicate that populations of the more sensitive gastropod species are unable to reproduce (OSPAR, 2004). In Faro region, where ship traffic is comparatively lower than in Lisbon and Setúbal, most of the offshore stations (14 in 16) had been exposed to TBT concentrations close to zero, which is the objective in the OSPAR hazardous substances Strategy (OSPAR, 2003b).

We can thus conclude that TBT pollution is undoubtedly a matter of concern in Lisbon and Setúbal, not only inside the estuaries where harbours are implanted but particularly in extensive areas of the adjacent continental shelves, regardless the massive dilution of contaminants that may occur in these deeper zones. This was also observed by Rato *et al.* (2006) in the north Portuguese coast. These authors also performed a survey on the imposex intensity of *N. reticulatus* inside the Ria de Aveiro and the adjacent continental shelve and found that imposex was spread over an extensive area outside the estuary down to 34-m depth (the VDSI ranged from 0 to 2.5). Hence, TBT pollution is spread over a considerable area of the Portuguese continental shelf outside the estuaries that embrace harbours, with effective risks to local ecosystems. Besides, some studies have shown the occurrence of imposex in whelks and organotin contamination of animals, such as molluscs, fishes, birds and mammals, in remote ocean regions from the coast around the world probably related to busy commercial shipping lanes (Tanabe, 1999; Tanabe, 2002; De Mora *et al.*, 2003; Strand and Jacobsen, 2005; Gomez-Arisa *et al.*, 2006). The extrapolation of the current findings to a global scale leads to the suspicion that the spatial magnitude of this pollution and associated ecological effects in marine waters is indeed very high, considering the ubiquity of the use of TBT antifouling paints all around the world. This reinforces the importance of the IMO global ban on the use of TBT antifouling systems on ships due to be in place by September 2008. Our results also form a substantial baseline on TBT pollution in the region with which to assess the success and time scales of recovery following the ban.

Acknowledgement

We would like to thank the crew of R/V ‘‘DIPLODUS’’ for their skilful handling of the boat and the gears. This work was developed under the research project POCI/MAR/61893/2004 financed by the FCT and by the POCI 2010, co-financed by FEDER. This work was supported trough a PhD grant (SFRH/BD/12441/2003) attributed by the Portuguese Foundation for Science and Technology (FCT). Financial support was also provided by the "Global COE (Centers of

Excellence) Program" from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan and Japan Society for the Promotion of Science (JSPS).

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CAPÍTULO 4

CHAPTER 4

Evolução Temporal do *Imposex* em *Nassarius reticulatus* (L.) ao longo da Costa Portuguesa: a Eficácia do Regulamento CE/782/2003

Temporal Evolution of Imposex in *Nassarius reticulatus* (L.) along the Portuguese Coast: the Efficacy of the EC Regulation 782/2003

Aceite para publicação na revista científica/Accepted for publication in:
Journal of Environmental Monitoring (DOI: 10.1039/B810188D)

Abstract

Imposex levels in *Nassarius reticulatus* (L.) were determined in 44 sites along the Portuguese coast in 2006 in order to describe spatial and temporal trends of TBT pollution in the area. The percentage of females with imposex across sites varied between 20 and 100, denoting the extent of this phenomenon throughout the Portuguese coast. The mean female penis length per site varied between 0.0-8.0 mm and the relative penis length index (mean female penis length x 100 / mean male penis length) attained a maximum value of 92%, i.e., female penis never surpasses the size of the male penis but nevertheless it can almost approach the male dimensions. The vas deferens sequence index ranged from 0.2 to 4.5 and the oviduct convolution index varied between 0.0 and 1.3 across stations. The penis growth, the vas deferens development and the oviduct convolution were all correlated and constitute visible signs of a global virilisation progression in females in response to the proximity of harbours that constitute the main TBT pollution sources. The results indicate that about 95% of the surveyed sites were still exposed to TBT water concentrations above the OSPAR Environmental Assessment Criteria. Nevertheless, signs of recovery are shown by the significant reduction of VDSI levels in 2006 in comparison to 2003, which points to the efficacy of the EC Regulation 782/2003 in reducing TBT pollution levels in the Portuguese coast.

Key words: *Nassarius reticulatus*, Imposex, TBT pollution, Temporal evolution, EC Regulation 782/2003

4.1 INRODUCTION

Throughout history numerous methods have been used to counteract the settlement of organisms on the ship hulls. The most successful antifouling coatings known to date are those based on tributyltin (TBT), which were firstly introduced in the 1960's (Hunter & Anderson, 2000). TBT paints have offered important economical benefits to shipowners, namely, fuel savings, extended dry-docking intervals and increased vessel availability due to less time spent in dry dock. Consequently, in 1996, for example, the TBT paints were used in 70% of the world fleet (approximately 27,000 ships) (CEFIC, 1996).

TBT was designed for toxic action at the ships surface but once released into the water it does not remain confined to the immediate vicinity of the ships since it is dispersed through the water where it rapidly adsorbs by biota and to suspended particles that later deposit onto sediments (Stewart & de Mora, 1992; ten Hallers-Tjabbes, 2000). Organisms can accumulate TBT by ingestion of contaminated food or via contact with contaminated water or sediments. The deleterious effects of TBT released by antifouling paints became evident in the beginning of 1970s as females of different gastropod mollusc species started to appear along the Atlantic coasts with male characters, a phenomenon coined as "imposex" by Smith (1971). In 1974 the oyster (*Crassostrea gigas*, Thunberg) culture at Arcachon (France) showed failures in spat recruitment, whilst the shell calcification anomalies caused a decline in the marketable value of the remaining stock (Alzieu, 2000). After the mid 1980s many other studies have described the TBT toxicity in organisms over a broad taxonomic spectrum, from bacteria to vertebrates, and its negative impacts at the individual, population and community levels.

Legislation to ban the use of organotin (OT) antifouling paints on small boats (<25 m) was introduced for the first time in France in 1982 and later in other European countries such as UK in 1987. These measures led to a reduction in imposex levels and clearance of oyster pathologies in many areas, accompanied by an amelioration of TBT contamination in the biota, water and sediments (Bailey & Davies, 1991; Evans *et al.*, 1995; Birchenough *et al.*, 2002; Reitsema *et al.*, 2002; Huet *et al.*, 2004). For example, in the Bay of Arcachon the oyster farming returned to normal: as early as 1983 for spatfall and 1984-1985 for shell anomalies (Alzieu, 1991) and in SW England the *Nucella lapillus* (L.) imposex and female sterility declined following the ban (Gibbs & Bryan, 1996). The European Union (EU) applied the above restrictive measures to the member states in 1989 (the Directive 89/677/EEC) by banning the retail sail or use of OT paints for pleasure boats (less than 25 m) and fish net cages. However, TBT pollution did not decrease at many EU areas, especially those subjected to large ship traffic. This was the case of Portugal: this country adopted the Directive 89/677/EEC in 1993 but by 2000 there was no recovery of imposex in gastropods throughout the coast (Barroso & Morreira, 2002; Barroso *et al.*, 2002a). In 2001, the International Maritime Organization (IMO) adopted the "International Convention on the Control of Harmful Antifouling Systems on Ships" (AFS Convention), which stated the prohibition of the application of organotins as biocides after January 1st 2003 and the total ban of its usage after January 1st 2008. Nonetheless, this convention could only enter in force 12 months after 25 States representing 25% of the world's merchant shipping tonnage have ratified it. These numbers were only achieved on 17th September 2007 with the 25th State ratification, representing a total of 38% of the world's merchant shipping tonnage (IMO, 2007). However, anticipating a slow ratification process and being aware of the urgent need to implement restrictive measures, the EU put in place the statements of the AFS-Convention in July 2003, through the EC Regulation 782/2003 (see also the Directive 2002/62/EU) to prohibit the application of OT compounds on all kinds of vessels flying the flag of a Member State.

The main objective of the present study is to evaluate the efficacy of the EC Regulation 782/2003 in reducing the TBT pollution in Portugal, using the gastropod *Nassarius reticulatus* (L.) as a bioindicator. This is achieved by assessing the levels of imposex of this species in 2006 along the Portuguese coast and by comparing these levels with those reported previously in 2000 and 2003 for the same sites. Additionally, it is intended to characterize the spatial trend of imposex intensity in several nassariid species along the Portuguese coast.

4.2 STUDY AREA

The study area corresponds to the Portuguese coast comprised between Vila Praia de Âncora (northern limit) and Faro (southern limit) (Figure 4.1), in a coastal extension of about 880 km.

The main Portuguese harbours lie along this study area and are located inside natural embayments, like estuarine systems. They may include commercial and fishing ports, marinas and dockyard facilities (Figure 4.1), which in most cases are close to each other. Table 4.1 summarizes relevant information regarding boat traffic and dockyard activity in the Portuguese coast. Lisbon harbour corresponds to the main national commercial port, followed (in terms of GTs) by Sines, Leixões, Setúbal, Aveiro, Viana do Castelo, Figueira da Foz and Faro. Fishing boats GTs at each harbour (Table 4.1) provide an estimate of the relative distribution of the fleet in the Portuguese coast and show that Aveiro harbour encloses by far the highest ship tonnage (four times higher than the second on the rank, Viana do Castelo).

Along the study area, there are 20 main marinas with an average berthing capacity (YBC) of approximately 300 yachts, although Lisbon and Portimão can berth, respectively, eightfold and twofold that number of vessels. There are also many small boat landings spread along the coast. The main dockyards in Portugal are located in the harbours of Viana do Castelo, Aveiro, Figueira da Foz, Peniche, Lisbon, Setúbal and Portimão (Table 4.1). The ship traffic in the main harbours of Portugal for 2000, 2003 and 2006 is presented in Figure 4.2.

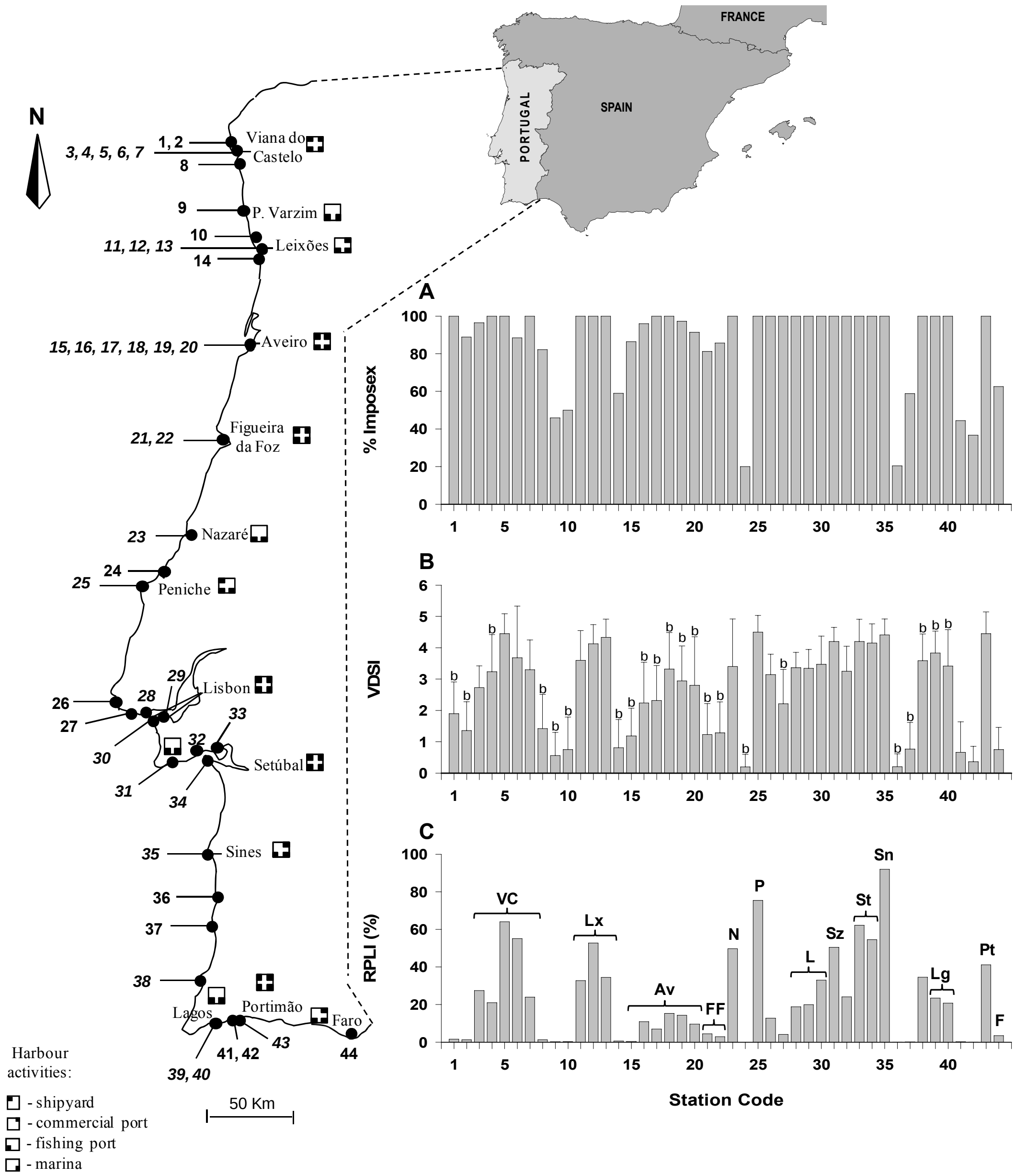

Figure 4.1 – *Nassarius reticulatus*. Map of the Portuguese coast indicating the sites (1-44) where specimens were collected and the location of the main harbours. Italic code numbers represent sampling stations located inside harbours. The graphic bars represent (**A**) percentage of female affected by imposex (%I), (**B**) vas deferens sequence index ($VDSI_5$) and (**C**) relative penis length index (RPLI) for each sampling station. Error bars correspond to 1 standard deviation. The b letters, on VDSI chart, represent stations where females exhibiting b-type VDS stages were found. The braces indicate the stations inside the habours: VC – Viana de Castelo; Lx – Leixões; Av – Aveiro; FF – Figueira da Foz; N – Nazaré; P – Peniche; L – Lisbon; Sz – Sesimbra; St – Setúbal; Sn – Sines; Lg – Lagos; Pt – Portimão; F – Faro.

Table 4.1 – Characterization of boat traffic and dockyard activity in the Portuguese coast: total number of commercial ships called at each port during 2006 and respective total gross tonnage stood (GTs) (T=tons), fishing boats GTs registered in 2006 (information obtained from the Doca Pesca site – www.docapesca.pt), as a parameter to estimate the relative importance of fishing boat traffic between harbours; local leisure boat traffic is classified according to the yacht number berthing capacity (YBC) of all marinas at each site; presence (P) of main dockyards in the harbours.

	Commercial Shipping		Fishing boats	Marinas	Dockyards
Harbours	Nº	GT/10^3 T	GT/10^3 T	YBC	presence
Viana do Castelo	211	926	8.2	307	P
Póvoa de Varzim	–	–	7.4	231	
Leixões	2725	20415	5.9	278	
Aveiro	1064	3141	34.5	270	P
Figueira da Foz	320	823	2.6	300	P
Nazaré	–	–	0.5	150	
Peniche	–	–	5.4	150	P
Lisboa	3335	35776	6.1	2335	P
Sesimbra	–	–	3.8	180	
Setúbal	1498	16202	1.9	150	P
Sines	1361	29893	2.4	230	
Lagos	–	–	1.9	462	
Portimão	65	1104	4.1	620	P
Faro	23	67	4.4	375	

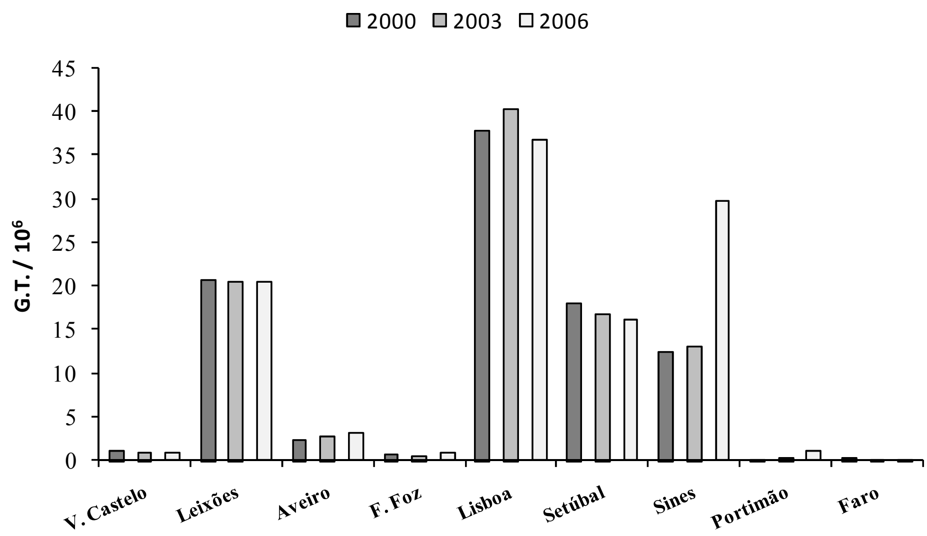

Figure 4.2 – Data regarding the gross tonnage (G.T.) of ships entered in the main harbours of the Portuguese coast in 2000, 2003 and 2006. Data obtained from INE – Instituto Nacional de Estatística – Statistics Portugal (www.ine.pt).

4.3 MATERIALS AND METHODS

4.3.1 Sampling and pre-treatment

Sampling of gastropods was performed between May and August 2006, from Vila Praia de Âncora to Faro. Sampling locations were selected in order to provide an extensive coverage of the mainland coast of Portugal, including the main national harbours (Figure 4.1; Table 4.2).

Geographical coordinates were determined with a mobile global positioning system (GPS) at each sampling site. The specimens were collected by hand at the intertidal shore and with baited hoop nets at sublitoral sites. The animals were maintained in aquaria for about 1-3 days. Whenever possible, 30 or more animals of each gender were analyzed per sampling station. Only adult animals were selected. They were narcotized using 7% $MgCl_2$ in distilled water. The shell height (distance from shell apex to lip of siphonal canal) was measured with vernier callipers to the nearest 0.1 mm. The shells were cracked open with a bench vice and the individuals were sexed and dissected under a stereomicroscope. Parasitized specimens were discarded from the analysis.

Table 4.2 – Data on *Nassarius reticulatus* collected along the Portuguese coast: number (N) of males (♂) and females (♀), mean (H) shell heights of males (♂) and females (♀) (mm), vas deferens sequence index ($VDSI_5$ and $VDSI_4$), average oviduct stage (AOS), mean female penis length index (mm) (PLI) and mean male penis length (mm) (MPL). Standard deviations relative to mean shell heights (H) are given as a percentage of the mean: (a) 0 to 5%; (b) 5 to 10 %; (c) 10 to 15%; (d) 15 to 20%; (e) 20 to 25%; (f) 25 to 30%; (g) 30 to 35%. For %I and RPLI values compare with Figure 4.1.

Station Code and Name	Coordinates (EUR 50)		♂(N)	H ♂	♀(N)	H ♀	$VDSI_5$	$VDSI_4$	AOS	PLI	MPL
1. Vila Praia de Âncora	41º 41.93 N	08º 51.94 W	34	22.52(c)	40	26.32(b)	1.90	1.90	0.00	0.19	11.38
2. Praia Norte	41º 41.85 N	08º 51.13 W	35	16.83(g)	53	21.43(f)	1.36	1.36	0.00	0.08	5.21
3. V. Castelo - Marina	41º 41.70 N	08º 49.20 W	37	22.30(e)	29	21.81(g)	2.72	2.72	0.00	1.46	5.30
4. V. Castelo - Marégrafo	41º 41.43 N	08º 49.71 W	35	20.52(f)	42	25.16(d)	3.24	3.10	0.15	1.74	8.27
5. V. Castelo - Estaleiro	41º 41.38 N	08º 50.01 W	34	23.32(d)	44	25.92(c)	4.45	3.95	1.25	7.99	12.47
6. V. Castelo - Cais	41º 41.34 N	08º 50.26 W	53	22.95(e)	25	25.41(d)	3.68	3.24	0.68	3.06	12.75
7. V. Castelo - Barra	41º 41.06 N	08º 50.24 W	12	23.28(b)	10	24.33(c)	3.30	3.20	0.22	5.70	9.94
8. Praia da Amorosa	41º 38.72 N	08º 49.31 W	36	19.73(e)	62	25.26(c)	1.42	1.42	0.00	0.12	9.04
9. Póvoa de Varzim	41º 23.18 N	08º 46.40 W	43	21.39(d)	61	21.68(f)	0.56	0.56	0.00	0.02	9.18
10. Praia de Leça	41º 12.21 N	08º 42.82 W	11	24.19(b)	8	22.87(d)	0.75	0.75	0.00	0.03	10.50
11. Porto de Leixões - Plat. 1	41º 11.42 N	08º 41.43 W	45	23.52(b)	50	22.81(c)	3.60	3.48	0.45	4.14	12.64
12. Porto de Leixões - Marina	41º 11.30 N	08º 42.24 W	43	22.62(c)	39	23.69(c)	4.13	3.87	0.46	6.44	12.20
13. Porto de Leixões - Plat. 2	41º 11.26 N	08º 41.89 W	4	23.76(b)	3	26.35(b)	4.33	4.00	0.00	4.75	13.75
14. Praia da Foz	41º 09.78 N	08º 41.10 W	27	23.73(b)	78	25.70(b)	0.81	0.81	0.12	0.07	11.55
15. Aveiro - Muranzel	40º 42.49 N	08º 42.25 W	23	27.96(b)	37	29.37(b)	1.19	1.19	0.00	0.05	12.92
16. Aveiro - São Jacinto	40º 39.48 N	08º 43.56 W	15	24.29(b)	25	25.38(b)	2.24	2.16	0.00	1.05	9.66
17. Aveiro - P. Com. Norte	40º 39.06 N	08º 43.76 W	14	23.69(c)	41	25.55(c)	2.32	2.29	0.00	0.41	5.57
18. Aveiro - Terminal Químico	40º 39.46 N	08º 42.74 W	26	25.23(b)	31	25.80(b)	3.32	3.16	0.00	1.45	9.45
19. Aveiro - Magalhães Mira	40º 38.65 N	08º 44.06 W	18	27.02(b)	36	27.38(b)	2.94	2.83	0.00	1.16	7.88
20. Aveiro - Barra	40º 38.71 N	08º 44.82 W	23	26.99(b)	35	27.17(b)	2.80	2.71	0.00	1.13	11.78
21. F. da Foz - Marina	40º 08.91 N	08º 51.67 W	34	24.56(c)	48	21.89(e)	1.23	1.23	0.00	0.15	3.28
22. F. da Foz - Estaleiro	40º 08.60 N	08º 51.55 W	19	25.73(b)	14	26.95(b)	1.29	1.29	0.00	0.21	7.04
23. Nazaré - Porto de Pesca	39º 35.04 N	09º 04.39 W	10	26.70(b)	5	26.79(b)	3.40	3.20	0.40	3.88	7.80
24. Foz do Arelho	39º 25.70 N	09º 13.39 W	27	20.41(c)	80	25.28(c)	0.20	0.20	0.00	0.00	10.56
25. Peniche - Porto de Recreio	39º 21.15 N	09º 22.52 W	50	22.00(b)	8	22.83(b)	4.50	4.00	0.57	6.59	8.74
26. Praia do Guincho	38º 43.74 N	09º 28.46 W	11	23.64(b)	21	25.46(b)	3.14	3.14	0.10	1.57	12.25
27. Praia das Avencas	38º 41.21 N	09º 21.27 W	15	20.50(c)	28	22.00(b)	2.21	2.21	0.00	0.43	10.54
28. Lisboa - Marina de Belém	38º 41.50 N	09º 12.50 W	48	22.45(c)	22	23.57(c)	3.36	3.36	0.00	2.01	10.67
29. Lisboa - Porto Brandão	38º 40.77 N	09º 12.29 W	27	24.03(c)	32	24.64(c)	3.34	3.34	0.00	2.74	13.70
30. Lisboa - Trafaria	38º 40.55 N	09º 14.09 W	26	22.58(c)	19	24.61(b)	3.47	3.42	0.11	3.53	10.69
31. Sesimbra - Porto de Pesca	38º 26.25 N	08º 06.76 W	9	18.68(b)	5	20.31(d)	4.20	4.00	0.00	4.82	9.56
32. Portinho da Arrábida	38º 28.58 N	08º 58.97 W	16	24.28(b)	28	26.07(c)	3.25	3.25	0.00	2.52	10.88
33. Setúbal - Lota	38º 31.17 N	08º 52.58 W	51	21.32(b)	25	20.72(b)	4.20	3.88	0.00	5.57	8.96
34. Setúbal - Tróia	38º 26.25 N	09º 06.76 W	30	21.45(b)	26	21.05(b)	4.15	3.88	0.12	4.38	8.18
35. Sines - Porto de Pesca	37º 57.28 N	08º 52.21 W	54	20.51(b)	17	20.66(b)	4.41	4.00	0.00	6.94	7.55
36. Vila Nova de Mil Fontes	37º 43.30 N	08º 47.25 W	24	21.47(b)	49	21.23(g)	0.20	0.20	0.00	0.00	7.36
37. Zambujeira do Mar	37º 33.20 N	08º 47.44 W	26	21.67(b)	34	22.95(b)	0.76	0.76	0.00	0.02	10.22
38. Praia da Arrifana	37º 17.82 N	08º 52.11 W	26	23.51(b)	39	24.37(b)	3.59	3.56	0.00	3.25	9.40
39. Lagos - Porto de Pesca	37º 06.30 N	08º 40.33 W	13	18.29(b)	24	19.96(c)	3.83	3.67	0.00	1.88	8.00
40. Lagos - Barra	37º 06.08 N	08º 40.15 W	21	20.09(b)	36	21.46(d)	3.42	3.33	0.00	2.08	10.00
41. Alvor - Aquacultura	37º 07.97 N	08º 37.48 W	26	22.12(b)	18	24.17(c)	0.67	0.67	0.00	0.02	9.08
42. Alvor - Barra	37º 07.22 N	08º 37.14 W	27	23.26(b)	30	23.81(b)	0.37	0.37	0.00	0.01	11.15
43. Portinho de Ferragudo	37º 07.48 N	08º 31.24 W	18	20.61(c)	11	29.91(c)	4.45	3.91	0.00	2.89	7.04
44. Ilha da Armona	37º 01.55 N	07º 50.40 W	9	21.23(c)	8	21.21(b)	0.75	0.75	0.00	0.27	7.71

4.3.2 Imposex analysis

The imposex parameters determined for each sampling station were the mean female penis length index (PLI), the relative penis length index (RPLI = mean female penis length x 100 / mean male penis length), the vas deferens sequence index (VDSI), the average oviduct convolution stage (AOS) and the percentage of females affected by imposex (%I). The penis length was measured using a stereomicroscope with a graduated eyepiece to the nearest 0.14 mm. The VDS stages in *N. reticulatus* were classified according to the scoring system proposed by Stroben *et al.* (1992a) but the computation of the VDSI for each site was based on the methodology proposed by Barroso *et al.* (2002a) ($VDSI_5$), i.e., stages 4 and 4+ were computed with the numerical values of 4 and 5, respectively, instead of a common value of 4, which provides a better discrimination of imposex levels between different sites or different dates; nevertheless, the VDSI values computed according to Stroben *et al.* (1992a) are also given in Table 4.2 ($VDSI_4$) to allow comparisons with other studies. The progressive oviduct convolution in females was ranked according to the three-stage scale (0, 1, 2) of Barreiro *et al.* (2001) and the average value (AOS) was assessed per each station. Sterility of *N. reticulatus* females will be addressed elsewhere. Imposex determination in *N. incrassatus* was performed using the same methodology described for *N. reticulatus* except that VDS was classified according to Oehlmann *et al.* (1998).

4.3.3 Statistical analysis

The statistical analysis performed in the current work was based on nonparametric methods. The correlation analysis refers to the Spearman correlation coefficient. The comparison between groups was based on the Mann-Whitney test for independent samples and on the Friedman test for paired samples. The adopted significance level was 5%.

4.4 RESULTS

Nassarius reticulatus is very abundant along the Portuguese coast and represents, by far, the most common nassariid occurring in the surveyed area. We attempted to collect other nassariids but only a sufficient number of *N. incrassatus* (Strőm, 1768) was collected by hand or baited hoop nets. Therefore, the available samples could only provide the assessment of imposex in *N. reticulatus* and *N. incrassatus*.

4.4.1 **Nassarius reticulatus *imposex***

The imposex levels of *Nassarius reticulatus* at the different sampling stations are summarized in Table 4.2 and Figure 4.1. Females with imposex occurred at all visited sites, denoting the extent of this phenomenon throughout the Portuguese coast. In fact, imposex (I%) affected between 20 and 100% of the females across the sampling site. PLI varied between 0.0-8.0 mm and RPLI attained a maximum value of 92%, i.e., female penis never surpasses the size of the male penis but nevertheless it can almost approach the male dimensions. $VDSI_5$ ranged from 0.2 to 4.5. Most females (88.6%) presented a-type VDS stages, i.e. with simultaneous penis development (Stroben *et al.*, 1992a). Females exhibiting b-type VDS stages ("aphalic condition") were less common (11.4%) but nevertheless they were spread across 22 sampling stations along the coast (see Figure 4.1). There was a highly significant correlation between the $VDSI_5$ and the Ln PLI ($r=0.94$. $P<0.001$) (Figure 4.3) and also between $VDSI_5$ and Ln RPLI ($r=0.93$, $P<0.001$) across stations, which means that the development of the vas deferens is accompanied by an increase of the penis length, both expressing the virilisation process in females. Another possible sign of female virilisation is the gonadal oviduct convolution, resembling the sinuous seminal vesicle of the males; the AOS index varied between 0.0 and 1.3 across stations. The average oviduct convolution stage estimated for all the females exhibiting a given VDS stage (0 to 5) is significantly correlated ($r=0.90$, $P<0.05$) with the VDS stage (Figure 4.4). Hence, the intensity of oviduct convolution and vas deferens and penis development were all correlated and constitute visible signs of a global virilisation progression in females.

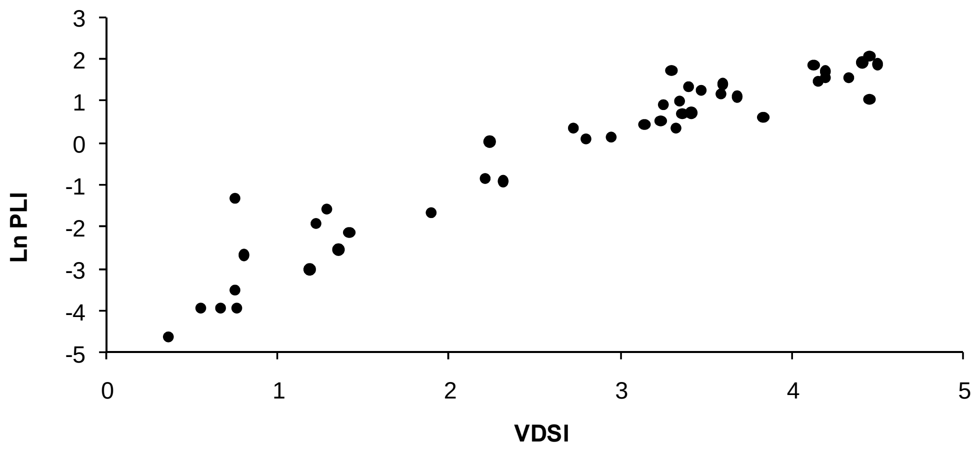

Figure 4.3 – *Nassarius reticulatus*. Relationship between the neparian logarithm of penis length index (Ln PLI) and the vas deferens sequence index ($VDSI_5$).

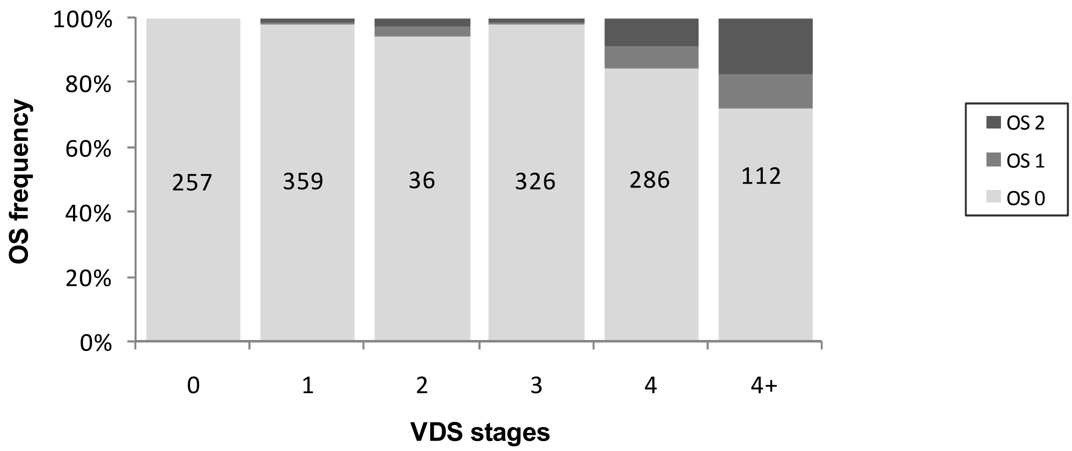

Figure 4.4 – *Nassarius reticulatus*. Relative frequencies of oviduct stages (OS) observed for each female VDS stage. The numbers represent the total female number observed for each VDS stage.

4.4.2 **Nassarius incrassatus** ***imposex***

Nassarius incrassatus samples were obtained in a relatively low number of sites, in some cases with a scarce number of animals, but still they could provide a coarse estimation of imposex levels in this species along the coast (Table 4.3). The percentage of affected females varied between 0 and 100%, VDSI between 0.0 and 3.5, PLI between 0.00 and 1.37 mm and RPLI between 0 and 31%. None of the observed females of *N. incrassatus* presented a convoluted gonadal oviduct. There was a significant correlation between imposex intensity in sympatric populations of *N. incrassatus* (only samples with 6 or more females were considered) and *N. reticulatus* for VDSI ($r=0.81$, $P<0.015$), PLI ($r=0.78$. $P<0.025$) and RPLI ($r=0.79$, $P<0.025$) but all imposex parameters presented lower values in N. *incrassatus*.

Table 4.3 – Data on *Nassarius incrassatus* collected along the Portuguese coast: station code, number (N) of males (♂), mean shell heights of males (H ♂, in mm), number (N) of females (♀), mean shell height of females (H ♀, in mm), percentage of females affected by imposex (%I), vas deferens sequence index (VDSI), average oviduct stage (AOS), mean female penis length index (mm) (PLI), mean male penis length (MPL) and relative penis length index (%) (RPLI). Standard deviations relative to mean shell heights (H) are given as a percentage of the mean: (a) 0 to 5%; (b) 5 to 10 %; (c) 10 to 15%.

Station code	♂(N)	H ♂	♀(N)	H ♀	%I	VDSI	AOS	PLI	MPL	RPLI
2	3	12.15(b)	7	11.85(b)	0	0.00	0.00	0.00	10.67	0
9	6	11.65(b)	4	10.54(c)	0	0.00	0.00	0.00	11.00	0
10	20	12.76(b)	20	12.72(b)	0	0.00	0.00	0.00	7.43	0
14	22	11.45(c)	29	10.98(c)	3	0.03	0.00	0.00	8.44	0
17	3	11.49(c)	4	11.02(c)	25	0.25	0.00	0.20	6.67	3
23	14	12.34(b)	12	11.86(c)	100	3.50	0.00	1.37	4.45	31
27	2	12.79(c)	2	12.58(a)	0	0.00	0.00	0.00	12.00	0
29	19	13.92(b)	14	13.59(b)	71	0.71	0.00	0.44	7.31	6
33	23	12.07(b)	22	12.09(b)	73	0.73	0.00	0.52	4.74	11
34	23	12.37(b)	33	12.46(a)	15	0.15	0.00	0.05	1.71	3

4.4.3 Spatial evolution of imposex in **Nassarius reticulatus**

Due to the low abundance of other nassariids species, *N. reticulatus* is the only one used to describe spatial trends of imposex intensity along the Portuguese coast. There is an evident relationship between the imposex levels in *N. reticulatus* and the proximity of harbours enclosing potential TBT contamination sources such as commercial and fishing ports, dockyards and marinas. The highest values of $VDSI_5$ (>4) were found at stations located inside the harbours of Viana do Castelo (Stn. 5), Leixões (Stns. 12 and 13), Peniche (Stn. 25), Sesimbra (Stn. 31), Setúbal (Stns. 33 and 34), Sines (Stn. 35) and Portimão (Stn. 43). The lowest values of $VDSI_5$ (<1) occurred at sites distant from harbours, either on the open shore (Stns. 9, 10, 14, 24, 37) or inside estuarine systems (Stns. 36, 41, 42, 44). There were no pristine sites with unaffected females (see Figure 4.1A), which probably due to the ubiquitous presence of small fishing and leisure boats and water current transport of TBT from contaminated areas. Considering all the surveyed sites, a highly significant difference (Mann-Whitney test, statistic=125.0, $P < 0.001$) can be observed in the $VDSI_5$ between two main groups of stations: distant from harbours (Stns. 1-2, 8-10, 14, 24, 26-27, 36-37, 41-42, 44) and close to harbours (remainder 30 stations). When the analysis is focused on a single region for which there is a reasonable number of sampling stations around a specific harbour, a clear increasing gradient of imposex is evident on approaching the harbour (Figure 4.1). For instance, at Viana do Castelo the lowest imposex levels occurred at stations outside the estuary in the open coast (Stns. 2, 8) whereas the highest levels were registered in the harbour inside the estuary (Stns. 3-7). Similar trends were also observed around Leixões and Lisbon (Figure 4.1B). The RPLI values presented the same general trend described above for $VDSI_5$, which is expected since both parameters were significantly correlated to each other (Figure 4.3). In fact, taking again Viana do Castelo as an example, the highest values of RPLI were found inside the estuary (Stns. 3-7) and the lowest values occurred outside the estuary (Stns. 2, 8). Once again the same pattern was observed for Leixões and Lisbon harbours (Figure 4.1C).

4.4.4 Temporal evolution of imposex in **Nassarius reticulatus**

Temporal comparisons of $VDSI_5$ in *N. reticulatus* for common sites sampled in 2000, 2003 and 2006 are shown in Table 4.4. This comparison allows the assessment of the temporal evolution of TBT pollution in recent years, as the imposex analyses were performed on the three occasions using identical methods. The comparison included the samples collected in the three dates in 19 stations using $VDSI_5$ as variable. The geographical locations of these stations were all the same for these three years, thus it was considered that the three samples were paired due to location reasons. The Friedman test (Conover, 1999) indicated that there was a highly significant decrease in the $VDSI_5$ levels (statistic=16.9, $P<0.001$). Multiple comparisons within the Friedman test were performed between these years concerning the levels of this variable and they showed a significant reduction of the $VDSI_5$ (statistic=20, $P<0.001$) between 2003 and 2006, although there was no statistical difference between the $VDSI_5$ levels (statistic=5, $P=0.279$) in the years 2000 and 2003.

Table 4.4 – Sampling stations code and respective $VDSI_5$ from 2000, 2003 and 2006, used for the assessment of temporal evolution of imposex. Data from 2000 and 2003 were published, respectively, by Barroso *et al.* (2002a) and Sousa *et al.* (2005).

Sation code	$VDSI_5$		
	2000	**2003**	**2006**
2	2.50	2.72	1.36
5	4.50	4.88	4.45
8	1.70	2.20	1.42
12	4.40	4.36	4.13
13	4.60	4.45	4.33
14	2.50	1.30	0.81
16	2.60	2.45	2.24
17	4.30	4.21	2.32
19	3.30	3.87	2.94
20	2.70	3.00	2.80
25	4.90	5.00	4.50
26	3.40	3.27	3.14
27	4.40	4.08	2.21
30	4.90	4.85	3.47
33	4.90	4.32	4.20
34	4.60	4.56	4.15
36	0.50	0.73	0.20
37	0.60	0.39	0.76
38	0.70	3.09	3.59

4.5 DISCUSSION

The current study shows that the netted whelk *N. reticulatus* is a common gastropod in the Portuguese coast. Specimens were found in large quantities in sandy or muddy sediments of sheltered and rocky shores, particularly inside estuarine systems. Despite the abundance in most of the sites along the coast, in some visited stations the species was scarce or even absent, especially in the southern coast. Nevertheless, the stations where the species was present provided a good monitoring coverage of the Portuguese shore, comprising the main harbours of both western (Viana do Castelo, Leixões, Aveiro, Figueira da Foz, Lisbon, Setúbal and Sines) and southern (Portimão and Faro) coasts. Other nassariids were collected during this survey, namely *N. pygmaeus* (Lamarck, 1822), *N. nitidus* (Jeffreys, 1867) (see Rolán & Luque, 1994) and *N. incrassatus*, but they were far less abundant than *N. reticulatus*. Although *N. incrassatus* is a suitable bioindicator (Oehlmann *et al.*, 1998), the current work shows that this species is less adequate than *N. reticulatus* for TBT pollution assessment along the Portuguese coast due to its poorer abundance and lower sensitivity. Other gastropods proposed in the literature as TBT pollution indicators, namely *N. lapillus* (L.), *Ocenebra erinacea* (L.), *Ocinebrina aciculata* (Lamark), *Littorina littorea* (L.) and *Hydrobia ulvae* (Pennant), do occur along the Portuguese coast and were also sampled in the current survey (to be published elsewhere). But among all these species *N. reticulatus* is by far the most abundant and ubiquitous in mainland Portuguese waters and should be regarded as the key bioindicator for national TBT pollution monitoring purposes.

It is well established that imposex is a fairly specific and dose dependent response to TBT pollution (Huet *et al.*, 2004). In *N. reticulatus* this relationship is supported by laboratory experiments (Stroben *et al.*, 1992b; Bettin *et al.*, 1996; Barroso *et al.*, 2002b) and by field evidence of correlation between imposex and TBT female body burdens, whether in the same area of the current survey (Barroso *et al.*, 2002a; Sousa *et al.*, 2005) or in other coastal areas such as Spain (Barreiro *et al.*, 2001), France (Stroben *et al.*, 1992a) and Britain (Bryan *et al.*, 1993). This fact allows the use of imposex per se as a reliable biomarker for the assessment of spatial and temporal trends of the level of TBT pollution, exempting the need to perform TBT determinations in the tissues or in the environment. The current work and the above studies have shown that imposex in *N. reticulatus* is expressed by three visible morphological changes: the development of a vas deferens, the growth of a penis and the convolution of the gonadal oviduct.

RPLI is a useful index to describe spatial trends of imposex in the sense that it provides very interesting images of spatial gradients around hotspots of pollution along the coast (see Figure 4.1C). However, indices based on "penis length" must be regarded with some caution because the current survey denotes the occurrence of aphallic females (imposex affected females lacking a penis) in half of the 44 stations sampled. As these females are computed by this index as "zero" when in fact they exhibit imposex, the RPLI may incorporate some bias. Moreover, the male penis size depends on the testis maturation state, which varies along the year and forces RPLI to vary according to the sampling season (Barroso & Morreira, 1998). Hence, for "inter-site" or "inter-date" statistical comparisons we consider that VDSI is a more reliable index to be used. This index integrates the development of the vas deferens and the initial development of the penis and it does not change seasonally. However, the VDS scales that are applied by different authors for *N. reticulatus* differ in what regards to the maximum numerical value that VDS attains after VDS stage 4. Some authors seek for a coherent standardization of VDS classification among different prosobranch species (see Stroben *et al.*, 1992b and Ohelman *et al.*, 1998) so that VDS=5 means that the vulva is blocked whereas VDS=6 corresponds to a stage when aborted capsules accumulate inside the capsule gland as a consequence of the vulva closure (as originally defined by Gibbs *et al.*, 1987, for *Nucella lapillus*). *N. reticulatus* females with aborted capsules have already been found in the European coast (Huet *et al.*, 1995; Barreiro *et al.*, 2001; Barroso *et al.*, 2002a; Sousa *et al.*, 2005) but there are still no experimental evidences that such conditions are a direct consequence of vas deferens development in this species and so, according to these criteria, the maximum possible score attained in *N. reticulatus* cannot exceed the value of 4 (corresponding either to VDS stage 4 or 4+). Other authors attempt to increase the resolution power of the index in order to improve the discrimination between different polluted sites, independently of what happens in other species, and the score can attain a maximum value of 5 (corresponding to 4+ according to Stroben *et al.*, 1992a) (Barroso *et al.*, 2002a) or 4.5 (Barreiro *et al.*, 2001) as the vas deferens grows beyond the vulva. Regardless which criteria is used there must be a special caution to provide VDSI values that can compare to results obtained by different authors. Hence, in the current work we apply $VDSI_4$ (stages 4 and 4+ are computed as 4) according to Stroben *et al.* (1992a) (Table 4.2) in order to compare our results with the OSPAR provisional assessment criteria (see below) and to make the data available to other authors but, on the other hand, we use $VDSI_5$ (stage 4 is computed as 4 and stage 4+ is computed as 5) to increase the power of the spatial and temporal evolution analysis in the surveyed area.

The current study shows that the higher levels of imposex are found inside or in the vicinities of harbours. This trend was also registered in previous studies (Barroso *et al.*, 2002a; Sousa *et al.*, 2005), strengthening the concept that areas in the proximity of harbours are "hotspots" of TBT pollution. In fact, Portuguese harbours enclose commercial/fishing ports and marinas where many boats are gathered and are sources for TBT contamination to the environment through the leaching of this compound from antifouling coatings into the water. Besides, most of them contain ship/boat construction and repairing dockyards. At these facilities boats are painted or repainted and the old layer of paint is removed from the hull, which results in slurry of wash-down water potentially contaminated with antifouling compounds and paint particles, which may represent a very important source of TBT input in the local (Birchenough *et al.*, 2002; de Mora *et al.*, 1995). Selecting the VDSI as a representative index of imposex, the current work reveals a highly significant difference in the values of $VDSI_5$ between stations distant and stations close to harbours, providing evidence of imposex progression on approaching the harbours. Considering the specificity of imposex as a TBT pollution biomarker, which provides robust information of TBT exposure at a given location, the results of this survey indicate that most of the sites analysed are still highly contaminated by TBT. In fact, OSPAR has developed provisional assessment criteria to evaluate monitoring data on TBT-specific biological effects related to the existing Environmental Assessment Criteria (EAC) for TBT (OSPAR, 2004). According to these criteria, among the 44 stations surveyed in the Portuguese coast only two stations have been exposed to TBT concentrations below the EAC derived for TBT ($VDSI_4$ <0.3); thirteen stations fall into class C (0.3 < $VDSI_4$ <2.0), which means that they have been exposed to TBT concentrations higher than the EAC and there is a risk of adverse effects, such as reduced growth and recruitment in more sensitive species, caused by long-term exposure to TBT; eighteen stations are ranked in class D (2.0< $VDSI_4$ <3.5) indicating that TBT exposure directly affects the reproductive capacity of more sensitive species; the remaining eleven stations are included in class E ($VDSI_4$

>3.5), i.e., the populations of more sensitive species are unable to reproduce, with the majority of the females sterilized (OSPAR, 2004). This analysis shows that there is currently a high ecological impact caused by TBT on the marine ecosystems along the Portuguese coast.

Barroso *et al.* (2002a) and Santos *et al.* (2002) have found that the implementation of the European Directive 89/677/EEC (banning the use of organotins on ships under 25 m) was ineffective in reducing imposex in *N. lapillus* along the coast of Portugal, indicating that there was no decline of TBT concentrations in the environment. The current results demonstrate that imposex levels of *N. reticulatus* in 2006 are significantly lower when compared with those from 2003 (Sousa *et al.*, 2005). It is meaningful to point out that no significant differences were found when comparing imposex levels from 2000 and 2003 surveys. It must be noted there was no decline in ship traffic along this period along the Portuguese coast (Figure 4.2). It seems therefore that the total ban on the application of TBT antifouling paints on submerged structures imposed by the EC Regulation 782/2003 did have a significant favourable impact on TBT pollution in Portugal. We expect that this decline may become more evident in the near future as ships will not be allowed to circulate with OT antifouling coatings after September 2008.

In conclusion, *N. reticulatus* is a key indicator species for TBT pollution monitoring programs along the Portuguese coast. The coastal areas of Portugal are still heavily polluted by TBT, particularly around the harbours, as the imposex levels found in these sites are high. Nevertheless, the comparison of the current results with data collected in 2003 shows that imposex levels are decreasing. The EC Regulation 782/2003 seems to be effective but the rate at which TBT pollution and imposex will recover must be assessed through the continual monitoring of the Portuguese coast, for which the current work provides an important baseline.

Acknowledgement –This work was developed under the research project POCI/MAR/61893/2004 financed by the FCT and by the POCI 2010, co-financed by FEDER. This work was supported trough a PhD grant (SFRH/BD/12441/2003) attributed by the Portuguese Foundation for Science and Technology (FCT).

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CAPÍTULO 5

CHAPTER 5

Evolução Espacial e Temporal do *Imposex* em *Nassarius reticulatus* na Plataforma Continental Adjacente à Ria de Aveiro (NW Portugal): Avaliação da Eficácia do Regulamento CE/782/2003

Spatial and Temporal Trends of *Nassarius reticulatus* Imposex on the Continental Shelf off Ria de Aveiro (NW Portugal): Assessment of the Efficacy of the Regulation EC/782/2003

Abstract

Nassarius reticulatus imposex (imposition of male characters onto female prosobranchs) levels were assessed in 2006 at 57 sampling stations situated on the continental shelf adjacent to the Ria de Aveiro (NW Portugal) at depths between 5 and 30 m, in order to evaluate spatial and temporal trends of tributyltin (TBT) pollution in the area. Imposex levels are lower comparing to Ria de Aveiro (where the main sources of pollution are located) but nevertheless they indicate that, despite the expected massive dilution of TBT in the water, almost half of the surveyed sites have been exposed to TBT water concentrations above the OSPAR Environmental Assessment Criteria. However, there are signs of decreasing TBT pollution as demonstrated by the significant reduction of imposex levels in 2006 comparing to 2004 and 2005, which highlights the effectiveness of the Regulation EC/782/2003 that forbids the application of TBT antifouling coatings on all kinds of boats or ships from 1st July 2003. The age of the whelks collected in the present survey was estimated to be between 3 and 5.5 years and this study shows that females probably had developed imposex in a period of declining TBT pollution as a consequence of the application of the Regulation EC/782/2003.

Keywords: *Nassarius reticulatus*; Imposex; TBT pollution; Statoliths; Regulation EC/782/2003.

5.1 INTRODUCTION

Tributyltin (TBT) has been used since the end of 1960's as an active ingredient of antifouling paints, thus becoming the main source of TBT in aquatic systems (de Mora, 1996). Despite the initial scepticism, the recognition of TBT as a worldwide pollutant arouse mainly from the public and political impacts of publications describing the shell malformations in oysters in the Arcachon Bay (Alzieu *et al.*, 1981) and the decline of prosobranch gastropods in SW England due to imposex (Bryan *et al.*, 1986) – the superimposition of male characteristics onto female dioecious gastropods (Smith, 1971). In 1982 the French government prohibited the use of TBT antifouling paints in vessels less than 25m of length and the UK adopted similar restrictions in 1987. The increasing number of studies describing the noxious biological effects of organotins on non-target organisms in the 1980's led several countries to impose restrictions on the use of TBT in antifouling systems: in 1988 the United States and in 1989 Canada, New Zealand, Australia and many European countries through the EC directive 89/677/EEC (Evans *et al.*, 2000). In 2001, an International Convention on the control of harmful antifouling systems on ships (AFS-Convention) was adopted at a Diplomatic Conference held under the aegis of the International Maritime Organization (IMO). As an immediate follow-up to the AFS-Convention, the European Union (EU) adopted the Directive 2002/62/EC and later the Regulation EC/782/2003 that prohibited the application of organotin compounds on ships flying the flag of a Member State from 1st July 2003 and for the elimination of the presence of organotin compounds on ships after January 2008.

In Portugal, the use of organotin paints on small boats (<25 m) was banned in 1993. Surprisingly, no amelioration was detected in the imposex levels of *Nucella lapillus* (L.) and *Nassarius reticulatus* (L.) along the shoreline Portuguese waters during the following decade (Barroso & Moreira, 2002; Barroso *et al.*, 2002; Sousa *et al.*, 2005; Galante-Oliveira *et al.*, 2006) probably because large ships dominate the naval traffic and dockyard activities in Portugal. In this case, the global ban imposed in 2003 should be effective in reducing imposex, or otherwise other factors were causing the prevalence of this syndrome. For this reason a very special attention is nowadays focused on the imposex evolution in the Portuguese coast following 2003.

N. reticulatus has been used as an indicator species in TBT pollution monitoring programs throughout Europe (Stroben *et al.*, 1992; Bryan *et al.*, 1993; Barreiro *et al.*, 2001; Barroso *et al.*, 2002; Ruiz *et al.*, 2005; Sousa *et al.*, 2005; Rato *et al.*, 2006). In Portugal the majority of the sampling sites that have been chosen for the monitoring surveys include estuarine systems where the sediments are mainly constituted by muds. As muddy sediments may trap TBT for many years (Dowson *et al.*, 1996), the level of contamination in this compartment may remain unchanged after the global ban, which could putatively cause a delayed recovery of imposex in sediment dweller species. Therefore the monitoring surveys should also address populations that inhabit sandy sediment substrates of the continental shelves outside the estuaries, at least up to some kilometres away from the shoreline. Moreover, it is important to monitor the spatial and temporal trends of TBT pollution in these regions as they stand among the most biologically productive areas of the oceans and support high fishery harvestings. Rato *et al.* (2006) surveyed in 2004 and 2005 the imposex on *N. reticulatus* in the continental shelf adjacent to the Ria de Aveiro (NW Portugal) creating a baseline for long term TBT pollution monitoring. The current study aims to describe the spatial distribution of *N. reticulatus* imposex in the same region in 2006 and compare the current situation with that of previous years. The ultimate goal is to explore the usefulness of this species to assess spatial and temporal trends of pollution and to assess the effectiveness of recent international legislation in reducing TBT pollution in the area.

5.2. MATERIALS AND METHODS

5.2.1 Study area

Nassarius reticulatus was sampled in September 2006 on the shelf area adjacent to Aveiro (NW Portugal) between 5 to 30 m. Sampling was performed in 57 stations distributed along 12 transects (stations with the same latitude) positioned between latitudes 40º 38.00N and 40º 43.50 N (Figure 5.1). Transects were regularly spaced of 0.5 nautical miles and each transect included 4 to 6 sampling stations. Sampling consisted of a 5 minutes tow using two dredges, one on each side of a boat. The dredges were 0.64 m in width and hauled a net bag of 35-mm mesh size. A total area of 140 m^2 was surveyed at each sampling station. The positioning of the sampling stations was performed with a GPS and mapped using ArcGis software from ESRI (Redland, CA, USA).

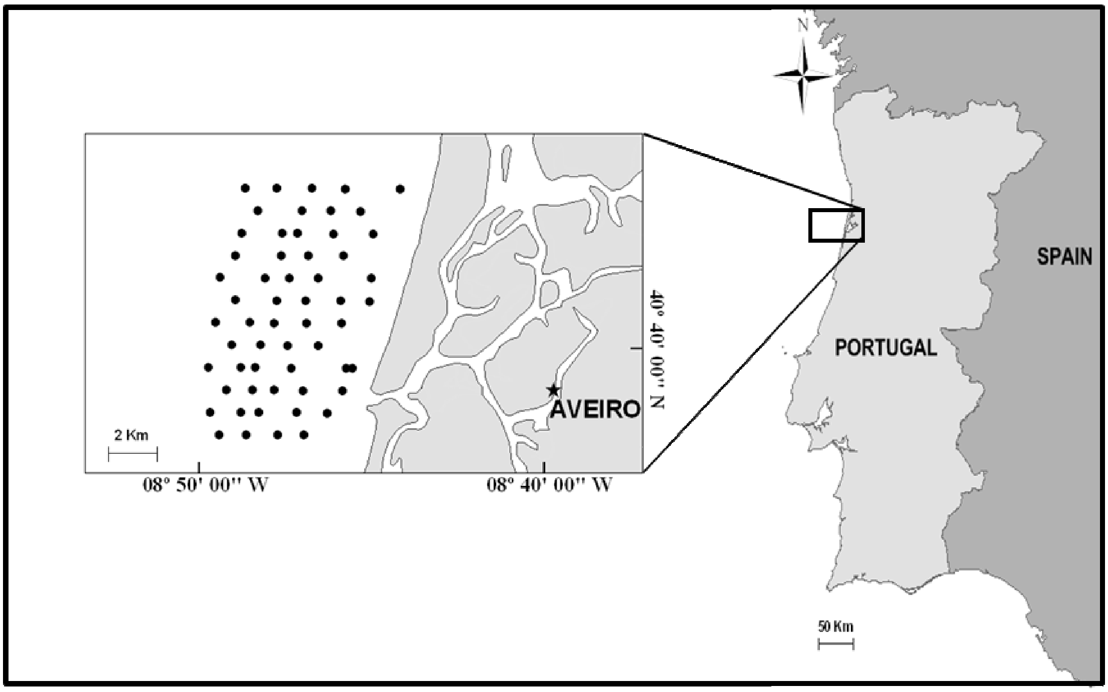

Figure 5.1 - Map showing the study area and location of the sampling sites. The study area comprises 57 sampling stations, distributed along 12 transects located between the latitudes 40º 38.00N and 40º 43.50 N.

The study area comprises an east-west navigational channel that runs from the traffic lane for commercial ships (far from the coastline) to the mouth of the Ria de Aveiro. This estuarine system is considered to be the main source of TBT pollution in the study area as it harbours important dockyards and fishing/commercial ports. Figure 5.2 shows the statistics regarding the number and gross tonnage of ships entered in Aveiro Commercial Port between 2000 and 2006. Within this period no major changes have been registered in the number of ships entered, but there was a slithly increase in their gross tonnage.

5.2.2 Imposex analysis

After collection, specimens were maintained alive in the laboratory and examined within 3 days. Adult animals (specimens presenting white columellar callus and teeth on outer lip) randomly selected from samples were narcotized with 7% $MgCl_2$ solution in distilled water. Shell height (distance from shell apex to siphonal canal lip) (SH) was measured with a Vernier calliper to the nearest 0.1 mm and then shells were cracked open with a bench vice. Individuals were sexed and dissected under a stereomicroscope. Percentage of females affected by imposex (%I), vas deferens sequence index (VDSI), penis length index (PLI) and relative penis length (RPLI: mean female penis length x 100/mean male penis length) were determined for each station. The VDSI was classified according to Stroben *et al.* (1992) and PLI was measured with a graduated eyepiece to the nearest 0.14 mm. In addition, the female oviduct convolution was also assessed according to the three-stage scale of Barreiro *et al.* (2001) and the average value (AOS) was determined for each station.

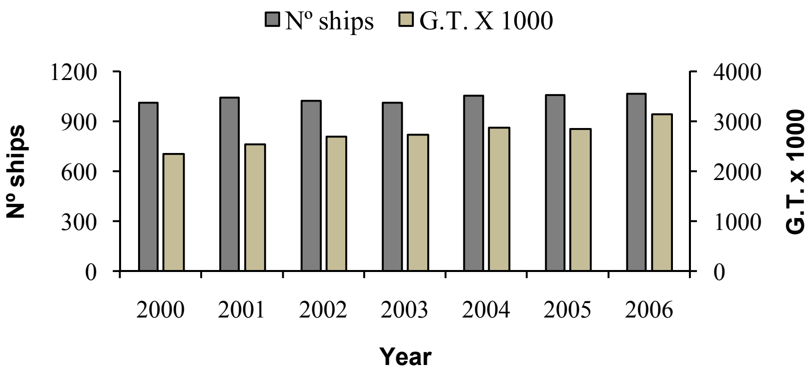

Figure 5.2 – Data regarding the number and gross tonnage (G.T.) of ships that entered in Aveiro Commercial Port between 2000 and 2006. Data was obtained from www.portodeaveiro.pt.

5.2.3 Age assessment

A sub-sample of 48 *N. reticulatus* specimens was randomly chosen from the surveyed area. These animals were similar to those used for imposex examination in what regards to shell height (SH) and shell morphology. Following collection, the whelks were maintained in aquaria for 2-3 days and then frozen at -20ºC until they were required for analysis. Upon thawing, the body of each whelk was extracted from its shell and dissected under a stereomicroscope to locate the statoliths. These were removed, cleaned in distilled water, dried at room temperature and embedded individually in small drops of epoxy resin, placed onto glass slides. The diameters of the statoliths and their growth rings were measured using a calibrated eyepiece graticule under the light microscope (OM), as counts of the statolith rings can provide an estimation of the age of *N. reticulatus* (Barroso *et al.*, 2005a; Chatzinikolaou & Richardson, 2007).The statoliths were then further examined under OM by grinding them down to their central nucleus on carborundum paper (1,200 grade) and polishing them with household metal polish to obtain an optimal smooth and polished surface. At this stage the growth rings were counted again in order to confirm the previous observations. The regression between statolith diameter (SD) and shell height was determined for a group of 123 specimens composed by the above 48 adults plus 75 smaller animals (mostly juveniles) presenting a wide range of shell sizes that were collected in the same area in previous sampling campaigns. The regression also included data regarding the SD and SH relationship in *N. reticulatus* veliger larvae reported by Barroso *et al.* (2005a) for the same area. This equation was used to convert measurements of statolith ring diameters into shell height.

Additional age information was obtained from the observation of the shell rings in the half end of the last whorl by the examination of the internal shell microgrowth banding patterns present in acetate peel replicas of polished and etched shell sections based on the technique described by Richardson *et al.* (1979) and Barroso *et al.* (2005b). Briefly, the shells were embedded in polyester resin and sectioned along the median growth axis. Sections were ground on sequentially finer grit and polished with household metal polish. The polished surfaces were etched for 5 minutes in 0.1 N HCl. Small acetate replicating strips (Agar Scientific) were placed in ethyl acetate for about 15 seconds, applied to the etched shell surfaces and dried at room temperature. The peels were removed from the shells and placed onto glass slides for examination under OM.

5.2.4 Statistical analysis

The statistical procedures used were the Spearman Rank Order Correlation, the Ordinary Least Squares multiple linear regression (OLS) and the Friedman test. The OLS regression was used to evaluate the effect of geographical coordinates in the VDSI (for each station). The Friedman test was used to compare the VDSI levels between the years 2004, 2005 and 2006 based on paired samples due to location reasons.

5.3 RESULTS

5.3.1 Imposex in Nassarius reticulatus

A total of 2478 specimens were analyzed, of which 1277 (52%) were females and 1201 (48%) were males. Imposex was spread over the study area as females affected by imposex were found in 48 of 57 stations sampled. Of the 354 females affected by imposex, 244 exhibited VDS stage 1, 12 presented VDS stage 2, 84 VDS stage 3 and 14 VDS stage 4. No animals were found presenting VDS stage 4+. Among females with imposex, 63 females presented the alternative "b-way" (all VDS stage 1), as described by Stroben *et al.* (1992). The %I ranged between 0 and 100% (Figure 5.3A), the VDSI varied between 0 and 1.67 (Figure 5.3B) and the PLI between 0 and 0.46 mm. The AOS and RPLI varied, respectively, between 0.0 and 0.36 and 0 and 7%. A significant correlation was found between VDSI and PLI (r=0.825, P<0.001).

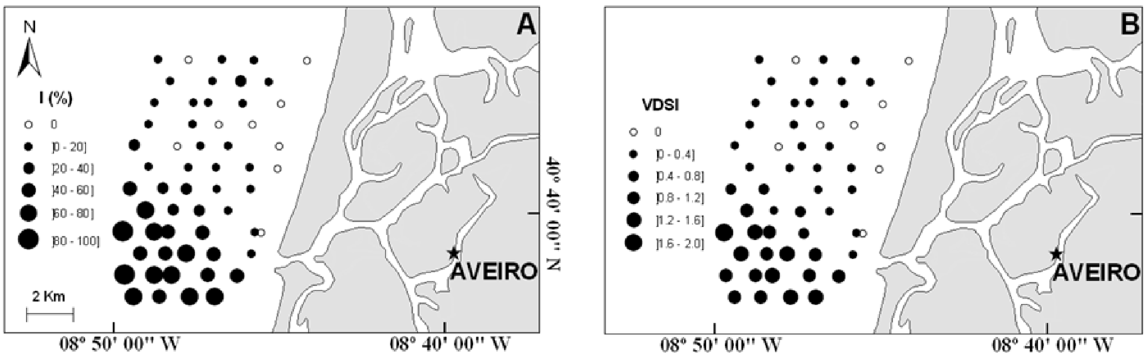

Figure 5.3 – *Nassarius reticulatus*. Map of the continental shelf adjacent to Aveiro indicating the spatial distribution of (**A**) imposex incidence (%I) and (**B**) vas deferens sequence index (VDSI).

5.3.2 Spatial evolution of imposex

The OLS analysis was performed in order to evaluate the relation between the mean imposex intensity at each site and geographical/physical variables. These included the position in the south to north direction along the latitude (expressed as the distance in km between a given station and the southernmost transect), the position in the east to west direction along the longitude (expressed as the distance in km between a given station and the coast line), the depth and the type of sediment. The latter covariate was expressed in two categories: presence or absence of large sediment particles (i.e., fine sands versus coarse sands, pebbles and shells) based on the qualitatively assessment of the sediment trapped during dredging operations. Hence, there were initially four covariates that were candidates to provide the best linear model to explain the intensity of imposex. The dependent variable is the intensity of imposex at each site. This variable is represented by VDSI because this is the most meaningful index to describe global imposex development as it expresses both the development of the vas deferens and the penis. Besides, females with imposex may not always exhibit a penis but the VDSI can still be scored ("b-way" vas deferens sequence). The analysis included all 57 sampling stations surveyed in 2006 that constituted the observations. We applied several model selection procedures, namely the stepwise regression and the backward elimination (Draper & Smith, 1998) that enabled us to select and keep the statistically significant covariates and put aside those that were not in any case statistically significant so that one gets the "best subset" regression. Hence, after evaluating the variables significance and the adjusted R^2 the covariates that proved to have a significant effect over the mean VDSI were latitude and longitude. The OLS linear regression model assumptions were checked, particularly in what concerns the normal distribution and the constant variance of the errors. None of these assumptions was rejected (so we could proceed with the analysis). One should have concern about the spatial correlation of the regression errors. In order to overcome this shortcoming of the OLS model one can use a great range of regression models that take into account the spatial nature of the data (see, for instance, Ripley (2004) and Fotheringham *et al.* (2006) for details about these statistical procedures). Nevertheless, the fact that the model includes covariates strictly related to the spatial location of each observation decreases a lot the statistical importance of the spatial correlation of the OLS regression errors because clearly the spatial covariates withdraw in a great extent the spatial component of the regression errors (see Johnston (1984) for an illustration). The results are shown in Table 5.1. The adjusted R^2 was 66.3% so that we can conclude that the geographical position of the sampling station explains about 2/3 of the VDSI variance. The P-value for the overall regression was 6.84E-14 indicating a highly significant fit (F=55.98). Regarding the estimated coefficients, the main conclusions are that for each kilometre from south to north the VDSI decreases about 0.124 and that for each kilometre from east to west the VDSI increases about 0.074. These estimates are highly significant as it is shown by the corresponding P-values (Table 5.1). It should be stressed that latitude has a higher impact (and also a greater significance) over the VDSI than the longitude.

Table 5.1 – *Nassarius reticulatus*. OLS multiple regression between imposex and geographical position of the sampling stations on the shelf adjacent to Aveiro in 2006. The observations were the 57 sampling stations. The variables were the distance from south to north along the latitude and the distance from east to west along the longitude (in km). The response variable is the VDSI observed at each sampling station.

	Constant	East/West	South/North
Coefficient	0.905	0.074	-0.124
Standard error	0.106	0.020	0.011
t-statistic	7.889	3.520	-10.074
P-value	1.49E-10	0.9E-3	5.26E-14

5.3.3 Temporal evolution of imposex

The purpose of the analysis is to compare *N. reticulatus* VDSI observed in 2006 with those reported by Rato *et al.* (2006) for 2004 and 2005, in 51 of the 57 sampling stations, for which the geographical locations are the same for the three years (Table 5.2). Therefore it was considered that the three samples collected at each site were paired due to location reasons. The Friedman test was used as a non-parametric alternative to the analysis of variance due to the restrictive assumptions of this last statistical procedure. The differences of the VDSI values (statistic=4.9; P=0.0096) among the years are significant, indicating that the levels of VDSI are not the same, so one made multiple comparisons between these years concerning the level of this variable (see Conover, 1999). Table 5.3

shows the statistics and P-values of those comparisons, which indicate significant differences between the year 2006 and the years 2004 and 2005. In the VDSI levels, there is statistical evidence that there was a reduction of the imposex levels in 2006 in the continental shelf adjacent to Aveiro in comparison to 2004 and 2005. Additionally, there was no statistical difference in the VDSI levels between 2004 and 2005.

Table 5.2 – Sampling stations code number and respective VDSI from 2006, 2005 and 2004, used for the assessment of temporal evolution of imposex. Data from 2004 and 2005 were published by Rato *et al.* (2006).

Nº	2006	2005	2004	Nº	2006	2005	2004	Nº	2006	2005	2004
1	1.37	1.97	1.33	17	0.67	1.38	1.32	36	0.08	0.09	0.05
2	1.37	1.76	1.38	18	1.03	1.95	1.39	37	0.01	0.00	0.00
3	1.13	0.96	1.43	20	1.75	1.33	0.93	38	0.00	0.23	0.05
4	1.00	0.81	0.88	21	0.29	1.09	0.45	39	0.29	0.24	0.14
5	0.85	2.5	1.50	22	0.42	0.75	0.92	41	0.00	0.05	0.1
6	1.09	1.7	1.58	23	0.29	0.39	0.61	42	0.17	0.24	0.19
7	1.29	1.71	2.48	24	0.97	0.81	0.69	44	0.00	0.04	0.00
8	0.94	0.91	1.43	25	0.2	0.33	0.23	45	0.06	0.00	0.10
9	1.00	1.14	0.78	26	0.08	0.22	0.44	46	0.05	0.09	0.20
10	0.15	0.59	1.44	27	0.36	0.46	0.09	47	0.12	0.08	0.18
11	1.2	0.25	0.54	28	0.45	0.38	0.07	48	0.10	0.25	0.00
12	1.5	1.29	1.13	29	0.5	0.41	0.47	49	0.10	0.13	0.24
13	0.82	1.83	2.00	30	0.00	0.13	0.00	50	0.21	0.15	0.13
14	1.21	1.71	1.79	31	0.03	0.10	0.05	51	0.10	0.12	0.16
15	0.00	0.31	0.61	33	0.14	0.23	0.43				
16	0.14	1.14	1.1	34	0.08	0.43	0.32				

Table 5.3 – Multiple comparisons within the Friedman test and corresponding P-values for the VDSI variable.

	Statistic	P-value
2004-2005	7.0	0.4455
2005-2006	20.5	0.0273
2004-2006	27.5	0.0034

5.3.4 Age of **Nassarius reticulatus**

The statoliths were classified in four types, according to the number of visible rings: 1R, 2R, 3R and 4R for statoliths with 1, 2, 3 and 4 rings, respectively (Table 5.4). The 2R type was further divided in two groups considering the position of the first annual ring (AR1). Rings were counted and measured in the OM and the mean diameter and standard deviation of the consecutive statolith rings are shown in Table 5.4. Among the 48 animals examined, 46% possessed statoliths with 4 rings (4R), 29% had statoliths with 3 rings (3R), 17% contained statoliths with 2 rings (2R) and 8% possessed statoliths with 1 ring (1R) (Table 5.4). There are reasons to suspect that the different number of statolith rings counted may not represent different ages but can simply result from the fact that some rings are not visible or were not formed in many individuals. In fact, the four statolith types occurred in individuals with similar shell sizes. This situation is evident in the 1R animals that possess a SH similar to the other groups and only present the metamorphic ring (MR) (see Table 5.4). This situation seems to repeat in the following statolith types where the latter rings were not observed. In this sense, 4R is the most representative statolith type as more rings are conspicuous and is the most common type. The diameters of these rings are very similar to those observed previously by Barroso *et al.* (2005a) for this species in the same geographical area (see discussion) and so the interpretation here performed is the same as the above authors. The relationship between statolith diameter and shell height is represented by the regression equation of Figure 5.4, which was used to estimate the shell height of the whelks at the time each statolith ring was produced. According to this equation we can interpret the 4R type statolith rings as follows: the nucleus has a mean diameter of 7.99 µm that corresponds to a shell size of about 0.7 mm and is formed during the embryonic development of *N. reticulatus*; the first ring (MR) has a mean diameter of 36.4 µm and is formed at a shell height of 1.4 mm when larvae metamorphoses and settles; the second ring (AR1) formed in the following winter with a diameter of 90.1 µm corresponding to a SH of 5.4 mm; the remainder rings (AR2 and AR3) formed in the next successive winters with mean diameters 124.1 µm and 143.4 µm corresponding to SH of 12.3 mm and 19.7 mm, respectively.

Table 5.4 – *Nassarius reticulatus*. Statoliths data, indicating the number of animals, statolith type, statolith ring diameters (µm), statolith diameter (µm) and shell height (mm).

Number of animals	***Statolith type***	***Statolith ring diameters (µm)*** *mean (standard deviation)*					***Statolith (µm)*** *mean (standard deviation)*	***Shell (mm)***
		Nucleus	*MR*	*AR1*	*AR2*	*AR3*	*SD*	*SH*
4	1R	8.13 (0.88)	36.41 (2.35)				159.84 (14.00)	29.30 (2.00)
5	2R[a]	7.25 (1.30)	37.00 (1.43)	87.50 (4.86)			160.13 (5.46)	29.22 (1.95)
3	2R[b]	8.33 (2.37)	35.42 (1.30)	131.67 (8.32)			158.75 (11.27)	30.61 (0.35)
14	3R	7.54 (1.16)	36.74 (2.63)	93.53 (8.09)	134.46 (12.25)		153.66 (10.32)	28.49 (2.28)
22	4R	7.99 (1.07)	36.38 (1.98)	90.10 (6.21)	124.13 (10.00)	143.44 (8.53)	157.28 (9.97)	29.39 (1.67)

Note: R – rings; 2R[a] and 2R[b] differ in the position of the AR1 ring; MR – metamorphic ring; AR – annual ring; SD – statolith diameter; SH – shell height.

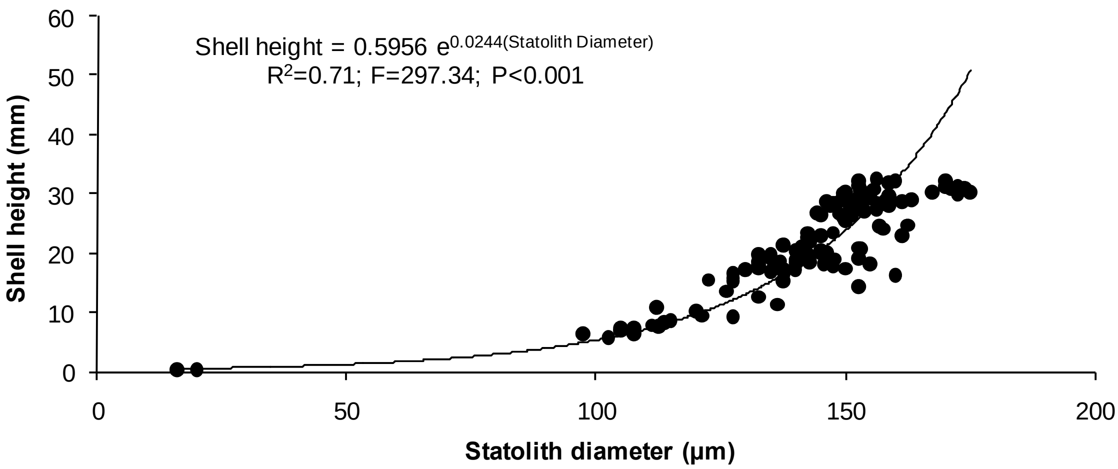

Figure 5.4 – *Nassarius reticulatus*. Relationship between statolith diameter and shell height with indication of the regression's equation and statistical parameters (N=123).

5.4 DISCUSSION

Nassarius reticulatus imposex is a sensitive biomarker for the determination of the level of environmental TBT pollution and has been widely used in TBT monitoring programmes throughout European shorelines (Stroben *et al.*, 1992; Bryan *et al.*, 1993; Barreiro *et al.*, 2001; Barroso *et al.*, 2002; Ruiz *et al.*, 2005; Sousa *et al.*, 2005; Rato *et al.*, 2006). The use of this species for monitoring spatial trends of TBT pollution in extensive areas of deeper continental shelves is a new approach that was applied with success by Rato *et al.* (2006) in the area adjacent to Aveiro. In fact, this is the only truly abundant gastropod species that occurs in this type of habitat in the Portuguese coast and that provides good samples size for statistical analysis of imposex data. While the above authors used this whelk for studying the spatial distribution of TBT pollution, the current work aims to explore the use of this indicator species for temporal trend analysis as well. We found that the spatial distribution of *N. reticulatus* imposex levels in 2006 was very similar to that previously described by Rato *et al.* (2006) for 2004 and 2005 in the same area (i.e., imposex is more intense to the south and west off the Ria de Aveiro), proving that imposex levels do not change erratically over space and time — a prerequisite when assessing spatial or temporal patterns of pollution. This also suggests that the animals present low mobility, as previously advocate by Rato *et al.* (2006). The OLS analysis performed in the current study confirms the occurrence of a significant decrease of VDSI from south to north (≈0.124 per km) and from west to east (≈0.074 per km). Rato *et al.* (2006) suggested that this spatial pattern occurs mainly because the Ria is the most important source of TBT pollution in the area and the residual current flow off the Ria is predominantly southward; besides, on the ebb, contaminated water from the Ria is pushed offshore by a jet flowing west and southwest from the mouth. This could explain the gradient observed in the south-north direction, but more studies are needed to clarify the observed west-east trend. One hypothesis for the latter trend is the eventual deposition of particles coming from the Ria de Aveiro in the western area. Another hypothesis could be the contamination derived from the deposition of dredged sediments removed from the Ria but, as the deposition site of dredged materials is located far away from the surveyed area (6 miles southwest off the estuary mouth) this hypothesis is unlikely to be the cause of the observed trend. Most importantly, the present study shows that *N. reticulatus* imposex is spread in the area with values of VDSI falling into OSPAR assessment classes A-C

(OSPAR, 2004). These values are low comparing to the shoreline situation inside Ria de Aveiro or other estuarine systems along the Portuguese coast (Barroso *et al.*, 2002), especially at sites close to ports where VDSI generally falls into classes D-E (OSPAR, 2004). Nevertheless, about 44% of the surveyed sites presented an VDSI>0.3 which means that the presence of TBT in the waters still exceeded acceptable levels in 2006 since VDSI higher than 0.3 indicates exposure to TBT concentrations above the OSPAR Environmental Assessment Criteria derived for TBT (OSPAR, 2004). This is a cause for concern as these sites are not immediately close to inputs of TBT (ports, dockyards and marinas) and are located in the sea at depths ranging from 5 to 30 m where high dilution and dispersion of TBT is expected to occur. Nonetheless, the situation shows signs of amelioration. In fact, a significant decrease of the imposex levels occurred in the surveyed area from 2004 to 2006 and from 2005 to 2006 as shown by the robust non-parametric Friedman test, indicating an overall decline in the level of TBT pollution. Considering that there was no decrease in the intensity of the ship traffic in the area along the last years (see Figure 5.2) this amelioration is most probably a consequence of the Regulation EC/782/2003 as this decline seems to be very recent because there are significant differences between 2005/2006 but not between 2004/2005.

It is expected that a decline of TBT level in waters will cause a reduction in imposex with some delay because imposex is an irreversible process. Hence, the rate of reduction of imposex in the population depends on how fast younger less-affected females substitute older ones that are more affected. If the adult specimens used in monitoring surveys belong to a mixture of very old cohorts it would be difficult to detect any short term imposex recovery but, on the contrary, if these specimens are not too old and are replaced every year by new recently matured animals this would produce fast imposex recoveries if TBT pollution is declining. The current work reveals a fast recovery of imposex levels in *N. reticulatus* in the continental shelf around Aveiro, which suggests that the latter scenario may apply to this case. This hypothesis is in fact corroborated by the age estimation analysis performed on the individuals used in the current survey. The rings in statoliths may be used to ascribe the age of *N. reticulatus* specimens (Barroso *et al.*, 2005a; Chatzinikolaou & Richardson, 2007). According to these authors, the first visible ring in the statolith is the ‘‘metamorphic’’ or “settlement” ring, which is formed at the time the larvae metamorphoses from the plankton and adopts a benthic mode of life. The other rings are formed every year as a result of the seasonal variation of the temperature. At Aveiro wider light increments are produced during the spring, summer and early autumn whereas darker narrow rings are formed between the late autumn and winter (a period henceforward designated as "winter"). The statoliths revealed that the larvae metamorphoses and settles with a SH of about 1.4 mm and then it grows up, in average, to 5.4 mm, 12.3 mm and 19.7 mm in the three following winters, respectively. These results are in accordance with Barroso *et al.* (2005a) as they found that statoliths of the largest whelks (>29 mm) at the same area contained three or four clearly defined rings, corresponding to SH of 1.1 mm, 4.6–5.3 mm, 12.0–13.5 mm and 18.5 mm, respectively. Neither the current work nor Barroso *et al.* (2005a) detected rings corresponding to a fourth and fifth years (with, respectively, 22.7–23.6 and 26.1–26.9 mm SH) that were estimated from shell height–frequency distributions obtained for *N. reticulatus* populations inside Ria de Aveiro by Barroso *et al.* (2005b). This is probably due to the lessening of the seasonal variation in the growth rate as the whelks' age, so rings become less discernible as the whelks increase in size. However, Barroso *et al.* (2005b) inspected the microgrowth banding of the shell edge lips of Ria de Aveiro whelks and noted that many large whelks contained up to six internal annual growth lines, meaning that the age of these whelks is likely to be up to 11 years old. In contrast, we did not find these rings in the shell lip, using the same method of these authors, and so the age of the adults used in this work is likely to be much lower. Chatzinikolaou & Richardson (2007) detected up to 7 annual growth rings in the statoliths of *N. reticulatus* from Anglesey (UK) corresponding to the following average SH: 6.0 mm, 12.01 mm, 16.3 mm, 19.0 mm, 20.9 mm, 22.2 mm and 24.5 mm. The first two rings are remarkably similar to those found in Aveiro but the remainder ones differ and show a lower growth rate for this higher latitude.

The age of *N. reticulatus* used in the current survey is difficult to determine exactly. Our best estimate is that 4R statoliths correspond to between 2 and 3 years, depending when settlement occurred. As there is at least one more growing season of about half an year between the time AR3 was formed and the date of the survey (September 2006), this accounts for a minimum age of 2.5-3.5 years. The margin between AR3 and the circumference edge of the statolith is of difficult analysis so that we are not certain if there are inconspicuous rings accumulated here. If we assume that these whelks grow at the same rate as those from inside Ria de Aveiro, then further winters had passed unnoticed when whelks were about 22.7-23.6 and 26.1-26.9 mm SH (Barroso *et al.*, 2005b), which gives two years more. The inexistence of annual rings in the outer lip of the shell suggests that the maximum age would not surpass 5.5 years. Hence, rather than assuming an exact age we prefer to suggest an interval of ages – a minimum of 2.5 years and a maximum of 5.5 years – within which individuals are more likely to fall. This is less than the maximum longevity reported for *N. reticulatus* for other sites in Europe: 10-15 years in Gullmar Fjord, Sweden (Tallmark, 1980), 17 years in Yealm Estuary, UK (Bryan *et al.*, 1993) and 11 years in Ria de Aveiro (Barroso *et al.*, 2005b). In the case that the animals collected in the current survey (in September 2006) had a maximum age between 3.5-5.5 years, they would have had an age of about 0.5-2.5 years when the ban took place in 2003. According to Barroso *et al.* (2005b) imposex attains its greatest development after 2 years of age in this species. Hence, the imposex of the females collected in the current survey would have developed imposex in a period of probable declining TBT water contamination as a consequence of the ban.

In conclusion, *N. reticulatus* is a good indicator species for monitoring TBT pollution in deeper areas of the Portuguese continental shelf as it is a very abundant species, unlike any other gastropod, and sufficiently sensitive to describe spatial patterns of pollution in extensive areas of the shelf. Besides, the population dynamics, at least in this area, permits to monitor short term changes in the levels of TBT pollution, which is useful to evaluate the effectiveness of recent TBT regulations. The *N. reticulatus* monitoring program developed in the last years for Aveiro indicates that a large area of the continental shelf is affected by TBT pollution, strongly influenced by the ship/boat traffic and dockyard activities in Ria de Aveiro, but there are evidences that this problem is lessening as a consequence of the recent ban on the use of TBT antifouling paints.

Acknowledgement –This work was developed under the research project POCI/MAR/61893/2004 financed by the FCT and by the POCI 2010, co-financed by FEDER. This work was supported trough a PhD grant (SFRH/BD/12441/2003) attributed by the Portuguese Foundation for Science and Technology (FCT).

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CAPÍTULO 6
CHAPTER 6

Evolução Temporal da Contaminação dos Sedimentos por Cobre e por TBT ao longo da Costa Portuguesa entre 2000 e 2006

Temporal Evolution of Copper and TBT Contamination in Sediments along the Portuguese Coast between 2000 and 2006

Abstract

Since the implementation of the EC Regulation 782/2003, TBT in antifouling paints (AFPs) has been replaced by other biocides, particularly copper. Due to this reuse of copper, some concern rose regarding the possible increase of environmental copper levels. In this study, sediment samples were collected in 2000 and 2006 and the concentrations of TBT and copper were assessed in order to analyse the temporal evolution of these contaminants in sediments. Copper concentrations varied between 1.5 and 244 µg/g dry weight (dw) in 2000 and between 10 and 143 µg/g dw in 2006. TBT levels ranged between <0.005 (detection limit) and 0.7 µg Sn/g dw in 2000 and between 0.021 and 0.7 µg Sn/g dw in 2006. Although there was a difference between TBT sediment concentrations when comparing stations close and distant from harbours (main sources of TBT), no difference was found in TBT sediment concentrations between 2000 and 2006. This indicates that sediments may act as long-term “reservoir”, which will slow down the TBT pollution recovery expected as consequence of the above mentioned EC Regulation. No difference was found in sediment copper concentrations between the same two years. Copper contamination has several sources which may conceal the possible increasing trend of copper environmental levels. The results also show a correlation between TBT and copper in sediments suggesting a common source but this relation may instead be due to a geographical coincidence of these contaminants sources. *Nassarius reticulatus* was also sampled in both years and copper body burden was determined to evaluate the potential use of this gastropod as bioindicator of copper contamination. In females, copper body burden varied between 83 and 1675 µg/g dw in 2000 and between 31 and 1170 µg/g dw in 2006. Males were only assessed in 2000 and their respective copper body burden ranged between 60 and 1803 µg/g dw. Again, no difference was found in copper body burden between the two years and no relation was found between copper body burden in *N. reticulatus* and copper sediment concentrations. It seems that copper body burden does not reflect copper environmental levels as this gastropod may efficiently regulate internal concentrations of this metal and, consequently, *N. reticulatus* might not be a suitable bioindicator of copper contamination.

6.1 INTRODUCTION

Tributyltin (TBT) was successfully introduced as a biocide in antifouling paints (AFPs) in the mid 1960's. These paints became rapidly widespread in the naval industry all around the world, e.g. in 1996 they were used in 70% of the world fleet (approximately 27,000 ships) (CEFIC, 1996). However, due to the negative impacts caused by TBT on non target species, the use of TBT AFP became progressively banned in many countries since the 1980's. In the European Union the TBT AFP were banned from small boats during the 1980's and 1990's and after 2003 their application was completely forbidden on any kind of ships through the EC Regulation 782/2003. As a consequence, other biocides have replaced TBT and, particularly, copper-based antifouling became more in use (Claisse & Alzieu, 1993; Voulvoulis *et al.*, 2002; Jones & Bolam, 2007; Schiff *et al.*, 2004; Srinivasan & Swain, 2007). Some studies have reported rising levels of copper in the environment because of its increased use in AFP. For example, in the Bay of Arcachon the mean concentration of copper in oysters rose from 80 to 150 $\mu g\ g^{-1}$ dry weight since the early 1980's till 1996, probably associated to the increased use of copper-based antifouling paints after the partial ban on TBT in 1982 (Claisse & Alzieu, 1993).

Copper is an ubiquitous element present in almost all compartments of the marine environment and also an essential element required by most organisms for the normal metabolic function, becoming toxic only when the organism is unable to regulate its excess (Jones & Bolam, 2007). In the aquatic environment, there are several potential sources of copper, including natural weathering of rocks and minerals containing copper, release of copper from sediment back into the water column and anthropogenic inputs such as sewage effluents, copper-based algaecides and pesticides, coal-producing industries, refining and smelting, mining, copper wiring, antifouling paints and hull maintenance activities (Srinivasan & Swain, 2007). Schiff *et al.* (2004) demonstrated that copper-based antifouling paints may leach approximately 4.0 $\mu g/cm^2/day$ or roughly 25g/month for a typical 9 meter boat. The use of new copper-based antifouling systems may have a massive impact in the marine ecosystem since it was estimated that AFP account for 15 x 10^6 kg/year of copper input into seawater (Blossom, 2002). Due to environmental concerns regarding the effect of copper on aquatic life, some countries have acted to reduce the use of copper-based antifouling paints (Showalter & Savarese, 2004). The European

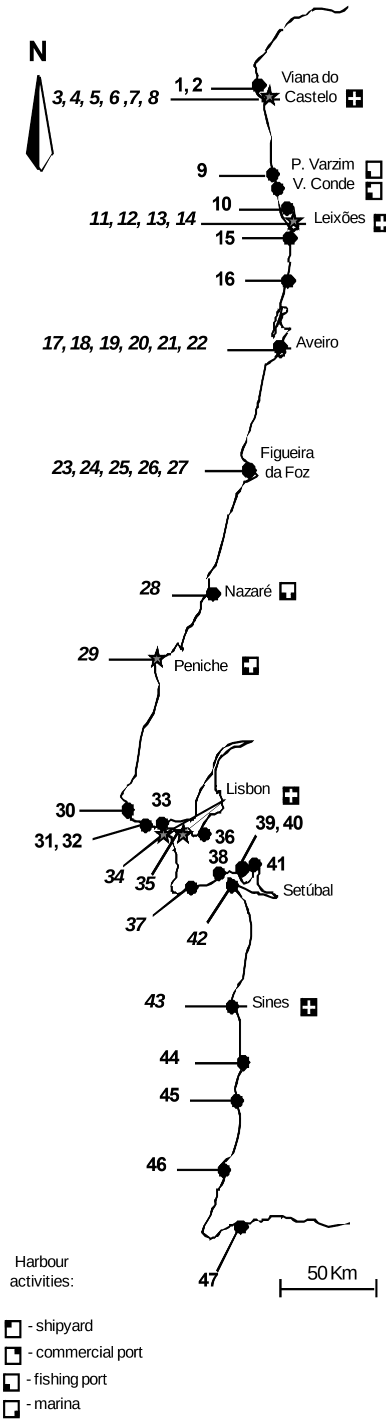
Commission gave copper a R50/R53 classification (Commission Directive 2008/58/EC), which states that this element is very toxic to aquatic organisms and may cause long-term adverse effects in the aquatic environment (Commission Directive 2001/59/EC). Sweden has instituted a ban of copper-containing antifouling coatings on pleasure crafts on its East coast in the Baltic Sea and has implemented a copper leach rate restriction from antifouling coatings on pleasure crafts on its West coast. Norway proposed to implement an *ecotax* in copper marine antifouling coatings. In 1999, the Dutch authorities banned the use of copper-containing antifouling paints on pleasure crafts (International Marine Coatings, 2007).

The objective of the present work is to assess the temporal evolution of copper and TBT sediment contamination along the Portuguese coast between 2000 and 2006, considering that the EU total ban on TBT AFP was implemented in 2003. The work also intends to study the temporal trend of *Nassarius reticulatus* copper body burden in the same period and its relationship with copper levels in the sediment, in order to evaluate the potential use of this gastropod as a bioindicator of copper contamination.

6.2 MATERIALS AND METHODS

6.2.1 Sampling and pre-treatment

Nassarius reticulatus and sediments were collected from May to July 2000 and from May to August 2006 along the Portuguese coast between Vila Praia de Âncora (North limit) and Praia da Luz (South limit) (Figure 6.1; Table 6.1). Sampling locations were selected in order to provide an extensive coverage of the West coast, including the main national harbours. Geographical co-ordinates were determined with a mobile global positioning system (GPS) at each sampling site. The whelks were collected by hand on the intertidal shore and with baited hoop nets at sublitoral sites. Only adult animals, i.e. those presenting white columellar callus and teeth on the outer lip, were used for the analysis. The shell height (distance from shell apex to lip of siphonal canal) was measured with vernier callipers to the nearest 0.1 mm. The shells were opened with a bench vice and the individuals were sexed under a stereomicroscope. About 20 females and 20 males were frozen separately at -20ºC, after removing the shell and the operculum. Samples were then homogenised, freeze dried and preserved at -20ºC until copper analysis was performed.



Nº	Sediments Cu (µg/g dw) 2000	Sediments Cu (µg/g dw) 2006	Sediments TBT (µg Sn/g dw) 2000	Sediments TBT (µg Sn/g dw) 2006	Sediments Organic Matter (%) 2000	Sediments Organic Matter (%) 2006	*Nassarius reticulatus* Females (µg/g dw) 2000	*Nassarius reticulatus* Females (µg/g dw) 2006	*Nassarius reticulatus* Males (µg/g dw) 2000
1							109.81	430.58	134.76
2	17.13		< d.l.		8.02		112.34	336.75	118.49
3	34.97	26.37	0.04	0.04	8.47	9.11	100.00		99.69
4	24.35		0.03		7.74		172.81		155.58
5	77.07	86.06	0.30	0.04	16.56	13.03	419.36	632.56	
6	131.03	53.92	0.57	0.32	10.06	9.29	1675.68		1803.49
7							142.38	287.38	165.71
8		10.74					218.75	289.50	207.72
9	34.56		0.01		34.17		125.70	109.38	60.15
10	2.81		0.01		11.21		241.57	110.12	185.26
11	244.83	82.43	0.47		11.85	15.23	198.14	224.84	184.17
12	11.68	82.32	0.22	0.19	16.65	10.22	314.02	314.11	458.35
13	3.48	57.42	0.14	0.15	11.01	15.55	204.54	170.21	210.93
14							137.95		240.72
15	39.69		0.02		12.00		301.60	144.93	257.12
16							235.18		349.22
17	19.30		< d.l.		5.21				
18	17.72	16.15	0.02	0.03	7.40	6.40	242.81	463.01	121.91
19	4.29	27.79	0.06	0.02	8.49	6.68	387.28	74.17	262.26
20	1.77		0.01		11.15		169.72	105.26	127.96
21	19.30	37.43	0.14	0.17	4.08	6.16	475.23	152.82	290.24
22	32.04		0.16		6.05				
23	2.97	30.97	0.06	0.03	11.07	9.91	293.55	31.09	130.52
24	30.94		0.03		8.67		121.45		117.33
25							87.68		101.91
26	37.23	36.92	0.03	0.02	7.89	9.15	170.89	48.11	90.21
27	29.95		0.12		8.04		832.85		682.55
28			0.73				216.10		163.11
29	8.32				11.62		162.43		
30			0.01				289.01	161.80	254.93
31	52.45		0.09		7.07				
32							282.04	146.24	233.08
33	39.08		0.05		6.58				
34		44.04	0.58	0.40	6.70	8.91	472.32	145.97	
35	2.18		0.03		8.99		248.52		253.70
36	45.49		0.47		8.24				
37							353.01		363.77
38							1449.15	1170.90	957.35
39	93.26	116.65	0.12	0.22	12.42	15.76			
40	5.59	143.01	0.10	0.66	12.76	15.25	118.18	226.61	186.49
41	14.07		0.51		9.43				
42	3.75		0.01		9.22		336.64	783.62	524.70
43				0.07			166.79	239.40	199.16
44	1.79		< d.l.		9.78		195.14		253.43
45	1.50		< d.l.		8.16		83.18		95.43
46			< d.l.				326.20		
47			< d.l.				329.78		148.50

Figure 6.1 – Map showing the study area, location of the sampling sites and data from 2000 and 2006 surveys indicating the station code (Nº), concentration of copper (µg Cu/g dw) and TBT (µg Sn/g dw) in the sediments, organic matter content of sediments (%), and female and male copper body burdens (µg Cu/g dw).

Table 6.1 – Station codes and respective geographical coordinates (Euro50) (Compare with Figure 6.1).

Station code and name	Coordinates (Euro50)	Station code and name	Coordinates (Euro50)
1. Vila Praia de Âncora	41º 41.93 N – 8º 51.94 W	25. Figueira da Foz – Barra	40º 08.86 N – 8º 51.90 W
2. Praia Norte	41º 41.85 N – 8º 51.13 W	26. Figueira da Foz – Estaleiro	40º 08.60 N – 8º 51.55 W
3. Viana do Castelo – Marina	41º 41.70 N – 8º 49.20 W	27. Figueira da Foz – Porto Pesca	40º 08.52 N – 8º 51.43 W
4. Viana do Castelo – Marégrafo	41º 41.43 N – 8º 49.71 W	28. Nazaré – Porto de Pesca	39º 35.04 N – 9º 04.39 W
5. Viana do Castelo – Cais	41º 41.38 N – 8º 50.01 W	29. Peniche – Porto de Pesca	39º 21.15 N – 9º 22.52 W
6. Viana do Castelo – Estaleiro	41º 41.34 N – 8º 50.26 W	30. Praia do Guincho	38º 43.74 N – 9º 28.46 W
7. Viana do Castelo – Barra	41º 41.06 N – 8º 50.24 W	31. Marina de Belém	38º 41.50 N – 9º 12.50 W
8. Praia da Amorosa	41º 38.72 N – 8º 49.31 W	32. Praia das Avencas	38º 41.21 N – 9º 21.27 W
9. Póvoa de Varzim	41º 23.18 N – 8º 46.40 W	33. Alcântara	38º 40.80 N – 9º 12.33 W
10. Praia de Leça	41º 12..21N – 8º 42.82 W	34. Lisboa – Porto Brandão	38º 40.77 N – 9º 12.29 W
11. Porto de Leixões – Plataforma 2	41º 11.42 N – 8º 41.43 W	35. Lisboa – Trafaria	38º 40.55 N – 9º 14.09 W
12. Porto de Leixões – Marina	41º 11.30 N – 8º 42.24 W	36. Alfeite	38º 40.47 N – 9º 08.70 W
13. Porto de Leixões – Plataforma 1	41º 11.26 N – 8º 41.89 W	37. Setúbal – Porto Comercial	38º 31.25 N – 8º 53.71 W
14. Porto de Leixões – Barra	41º 10.75 N – 8º 41.43 W	38. Setúbal – Porto de Pesca	38º 31.17 N – 8º 52.58 W
15. Praia da Foz	41º 09.78 N – 8º 41.10 W	39. Lisnave	38º 29.24 N – 8º 47.52 W
16. Espinho	41º 00.44 N – 8º 38.71 W	40. Portinho da Arrábida	38º 28.58 N – 8º 58.97 W
17. Aveiro - Muranzel	40º 42.49 N – 8º 42.25 W	41. Setúbal – Tróia	38º 26.25 N – 9º 06.76 W
18. Aveiro – São Jacinto	40º 39.48 N – 8º 43.56 W	42. Sesimbra – Porto de Pesca	38º 26.25 N – 8º 06.76 W
19. Aveiro – Porto Comercial Norte	40º 39.06 N – 8º 43.76 W	43. Sines – Porto de Pesca	37º 57.28 N – 8º 52.21 W
20. Aveiro – Barra	40º 38.71 N – 8º 44.82 W	44. Vila Nova de Mil Fontes	37º 43.30 N – 8º 47.25 W
21. Aveiro – Magalhães Mira	40º 38.65 N – 8º 44.06 W	45. Zambujeira do Mar	37º 33.20 N – 8º 47.44 W
22. Aveiro – Bruxa	40º 36.39 N – 8º 44.02 W	46. Praia da Arrifana	37º 17.82 N – 8º 52.11 W
23. Figueira da Foz – Marina	40º 08.91 N – 8º 51.67 W	47. Praia da Luz	37º 05.21 N – 8º 43.64 W
24. Figueira da Foz – Cais Comercial	40º 08.90 N – 8º 51.60 W		

Surface sediments (1 cm depth) were collected simultaneously from the same sites where *N. reticulatus* samples were obtained. At intertidal areas they were removed directly from the bottom whereas at sublittoral sites they were taken from the top of the sediment material collected with a van Veen grab. Separation of grain size, i.e., as the <63 µm or <100 µm fraction, has been recommended for chemical analysis because pollutants are mainly present in silt and clay particles (Langston and Spence, 1994) and also because it provides normalisation of the data regarding the grain size composition. Consequently, following collection the sediments were sieved with a 100 µm polypropylene mesh with water taken from each local. The sediments were allowed to settle, the supernatant water was decanted and the <100 µm fraction was frozen (-20ºC) in polyethylene bags. Later on, in the laboratory, the samples were homogenized, freeze dried and analysed for copper and TBT.

6.2.2 Copper quantification in **Nassarius reticulatus** *tissues and sediments*

For tissue analysis, approximately 200 mg of homogenized dry soft tissues of *N. reticulatus* were digested in a Teflon beaker and 4 mL of concentrated nitric acid were added. The beakers were sealed and heated to 60ºC overnight (12 hours). On the next day, the temperature was increased to 100ºC for 1 hour. After they were cooled down, then 2 mL of hydrogenous peroxide were added and the unsealed beaker contents were heated to 80º C for 1 hour. To ensure the full digestion of the samples, 1 mL of hydrogenous peroxide was added and the beakers were again heated to 80ºC for 30 minutes. After cooling down, the volume was brought to 100 mL with deionized water. For sediment analysis, about 100 mg of each dried sediment sample were digested in a Teflon beaker with a combination of nitric and perchloric acid in the ratio 1:3, plus 6 mL of fluoridric acid, at 100º C for 1 hour. After cooling down, the excess of fluoridric acid was neutralized with approximately 5.6 g of boric acid and the volume was brought to 100 mL with de-ionized water (Rantala and Loring, 1977).

All samples were digested in duplicate and, for quality assurance and control, three blanks were processed at each digestion and certified reference materials (sediments MESS-2 and PACS-2, dogfish muscle DORM-2 and hepatopancreas TORT-2 provided by Institute for Environmental Chemistry, Ottawa, Canada) were always digested with samples using the same procedures. To avoid possible contamination, all material was previously acid-washed (nitric acid followed by hydrochloric acid).

Copper concentrations were determined using graphite furnace atomic absorption spectrometry (Varian SpectrAA-800) with Zeeman background correction. Recalibration procedure was performed after 20 determinations to generate a calibration curve against which sample concentrations were calculated. Standard solutions were prepared from a commercial 1000 mg/L copper stock solution. Analytical blanks and certified materials were analyzed in the same way as the samples. The agreement between the obtained analytical results for the certified reference materials and its certified values was satisfactory, with the recoveries being always > 90%. The detection limits for copper in tissues was 0.16 μg/g dry weight (dw) for sediments was 0.14 μg/g dw.

6.2.3 TBT quantification in sediments

The quantification of TBT in sediments followed the methods based on Ward *et al.* (1981) that are fully described by Bryan *et al.* (1986). Briefly, three 0.5 g aliquots of each sediment sample were placed in 30 mL stoppered boiling tubes. A 0.2 μg standard of TBT was added to one sample and a standard of DBT to another; after shaking, they were left for 1.5 hours. Five mL of concentrated hydrochloric acid was added to the tubes, which were shaken in 30 minutes intervals. Following the addition of 5 mL of hexane, the tubes were placed on an automatic shaker for 15 minutes. Five mL of distilled water were added and, after swirling briefly, the tubes were centrifuged at 2000 rpm for 4 minutes. Tin was measured in a PerkinElmer 76B graphite-furnace attached to a PerkinElmer 603 atomic absorption spectrometer (Wellesley, MA, USA). A 1 mol/L sodium hydroxide solution was used to separate dibutyltin (DBT) from the TBT fraction; tin, as TBT, was subsequently measured in the NaOH-treated extracts, rendering a detection limit of 0.005 μg Sn/g dw.

6.2.4 Organic matter quantification

The organic matter content of the sediment was estimated from the loss weight after 4 hours on ignition at 450ºC (Head, 1985).

6.2.5 Statistical analysis

The data analysis was performed with the SigmaStat software (Systat Software, Inc., USA). Non parametric statistics were applied because the hypothesis of an underlying normal distribution of the data was rejected. Spearman Rank Coefficient was used for the correlations analysis. Mann-Whitney tests were used to test the differences of medians between two groups. Temporal trends were assessed by use of the Wilcoxon Match Pairs tests. Results were considered statistically significant for $P<0.05$.

6.3 RESULTS

6.3.1 Copper and TBT sediment concentrations

Copper concentration in sediments varied between 1.5 and 244 μg/g (dw) in the 2000 survey, and between 10 and 143 μg/g dw in the 2006 survey (Figure 6.1). The TBT concentrations ranged between <0.005 (detection limit) and 0.7 μg Sn/g dw in 2000, and from 0.021 to 0.7 μg Sn/g dw in 2006. There were no significant correlations between copper concentration ($r=0.24$; $P=0.112$) or TBT concentration ($r=0.18$; $P=0.231$) and the sediment organic matter content. Consequently, copper and TBT values were not normalized for organic matter content.

As both copper and TBT are used in ship antifouling paints we analyzed for possible differences in the level of sediment contamination between two main groups of stations - distant from harbours (Stns. 2, 8-10, 15-16, 30, 32, 40, 44-45, 47) and close to harbours (remainder 26 stations) – in 2000, as this year presents the most complete series of data. We found a significant difference (Mann-Whitney test, $U=203$, $P<0.001$) in the TBT sediment concentration between the first group (median=0.003 μg Sn/g dw) and the second group (median=0.11 μg Sn/g dw) of stations (assuming a value of zero for concentrations below the detection limit), denoting a clear increasing level of contamination in the proximity of harbours. There was no significant difference in copper sediment concentration between stations distant from harbours (Stns. 2, 8-10, 15-16, 32, 40, 44-45) and close to harbours (remainder 26 stations) but, nevertheless, the median levels were 2.2 fold higher in the second group (median=21.8 μg Sn/g dw) than in the first group (median=10.0 μg Sn/g dw). It is also interesting to note that levels of copper and TBT in the sediments across stations were significantly correlated to each other in 2000 ($r=0.48$; $P<0.01$) and in 2006 ($r=0.67$; $P<0.05$).

Copper and TBT concentration in sediments were reassessed in 2006 only for stations in the proximity of harbours (13 sites; see Figure 6.1) in order to perceive if the 2003 ban on the use of TBT antifouling paints could had produced a visible change in the copper and TBT sediment contamination scenario. Sediment TBT concentrations did not change significantly (Wilcoxon Test, $Z=0.784$; $P=0.470$) between 2000 and 2006 and the same applies for copper (Wilcoxon Test, Z=1.083; P=0.305), meaning that there was no significant temporal evolution in the levels of both contaminants in the sediments during the studied period of time. It should be mentioned at this stage that the organic matter content of the sediments did not change significantly in the above group of stations between 2000 and 2006 (Wilcoxon Test, Z=0.72; P=0.502); this fact and the lack of correlation between the contaminants and the organic matter in the sediments, mentioned earlier, indicate that this factor probably had a negligible effect on the temporal evolution analysis of copper and TBT levels.

6.3.2 Copper concentration in **Nassarius reticulatus** *tissues*

In 2000 copper concentrations in female tissues varied between 83 and 1675 μg/g dw whilst in males they ranged between 60 and 1803 μg/g dw (Figure 6.1). Female and male copper concentrations were significantly correlated across stations (r=0.779, P<0.001), suggesting similar processes of uptake and metabolism of this metal for both genders. In 2006 only female tissues were analyzed and the levels of copper varied between 31 and 1170 μg/g dw (Figure 6.1). There were no significant correlations between copper concentrations in the sediments and in the female tissues in 2000 (r=0.19, P=0.367) and 2006 (r=0.29, P=0.352); the same was observed in 2000 for males (r=-0.03, P=0.905).

We also analyzed the temporal evolution of copper concentrations in *N. reticulatus* female tissues between 2000 and 2006 for a group of 24 sampling stations and, alike sediments, there was no difference in the levels of this metal within this period of time (Wilcoxon Test, Z= 0.54; P= 0.597).

6.4 DISCUSSION

As the European Union banned the application of organotin paints in all kind of ships after 2003, TBT is now being replaced by other biocides, with relevance to copper. For this reason we attempted to perceive if there was any temporal evolution of TBT contamination in the Portuguese coast as a result of the above change of antifouling systems. Our results indicate that in the last years (2000 to 2006) there was no significant trend in the concentration of TBT in the sediments around the main harbours of the

Portuguese coast. This contrasts with the recent decline of imposex in *N. reticulatus* and *Nucella lapillus* observed in the coast of Portugal between 2003 and 2006 that is indicative of decreasing TBT pollution levels (Sousa *et al.*, 2007; Galante-Oliveira *et al.*, 2008; Rato *et al.*, *unpublished data*). This apparent contradiction could be related to the high persistence of TBT associated with sediments in comparison with its persistence in water. In estuarine waters the typical half-life of TBT is 6 to 7 days at 28°C and in oxic surface sediments is of a similar magnitude, however, in deeper anoxic sediments degradation is slower with half-lives varying from 1.9-3.8 years (Batley, 1996) to tens of years (Dowson *et al.*, 1996). Contamination of surface sediments by TBT from deeper layers cannot be discarded and so statistically significant trends may probably require longer periods of time to be detected. As antifouling paints represent the major source of TBT to the environment (Cheung *et al.*, 2003), TBT sediment contamination will certainly decrease in the future, but the question of how fast it will happen still remains. Our results raise the concern that sediments are acting as a long term 'reservoir' of TBT and will require management for a considerable period of time after the implementation of the EC Regulation 782/2003.

The current study also shows that there was no significant temporal evolution in the concentration of copper in the sediments around the main Portuguese harbours between 2000 and 2006, despite the expected increased use of copper AFP after 2003. These results require a different interpretation than the one used for TBT. In fact, while this organotin has a specific source of contamination, copper has a multitude of potential natural and anthropogenic inputs (Schiff *et al.*, 2004) that may obscure any trend derived from the increasing use of copper antifoulings. This could explain why in the current study a significant difference in TBT sediment concentration was found between sites close and distant from harbours but the same was not observed for copper. On the other hand, the fact that copper and TBT sediment levels were significantly correlated across stations may suggest a common contamination source. This was also observed in other geographical areas. For instance, in an assessment study of tin and butyltin species in estuarine superficial sediments from Gipuzkoa, Spain, Arambarri *et al.* (2003) found a good correlation between tin and copper that suggested a common pollution source. Recently, Harino *et al.* (2007) found a significant correlation between copper and TBT concentrations in sediment samples collected from Otsushi Bay, Japan. However, in our study we should take into account that harbours are located inside estuarine systems that certainly embrace other sources of copper and perhaps due to this geographical coincidence both contaminants are correlated to each other. Hence, there is a large uncertainty regarding the real contribution of the harbours to the actual levels of copper in the Portuguese coast. If the use of copper antifouling is increasing, we expect an increase of sediment copper levels in the near future, for which this study constitutes an important baseline for temporal trend analysis.

The current study also revealed no significant changes in the copper concentration in *N. reticulatus* tissues between 2000 and 2006. Also, there was no correlation between copper levels in sediments and in whelk tissues. It is possible that the netted whelk has the capacity to regulate internal levels of this metal. Unlike TBT, a minimum copper concentration is required for the organism healthy growth and this element is particularly important for gastropods as they need extra copper to meet their requirements of typical haemocyanin loads (Cheung and Wong, 1999). At high concentrations metals can become toxic for living organisms and to avoid the toxicity of metals aquatic organisms may use different strategies such as the limitation of the metal uptake, the increase of metal excretion or the metal immobilization within tissues and/or shell (Ahearn *et al.*, 2004). Hence, in some species, the metal concentrations in tissues may not reflect environmental levels. According to our data this appears to happen with *N. reticulatus* and so this species is probably a bad bioindicator of copper sediment contamination. However, Kaland *et al.* (2003) exposed *N. reticulatus* in the laboratory during 20 days to 50 µg/L Cu and noted increased levels of copper in the hepatopancreas, intestine, foot and gills, in comparison to the control. The gills appeared to be the main target organ with a fivefold accumulation over control values. The apparent contradiction of both findings could be explained by several factors, i.e., different bioavailability of copper in each situation (water *versus* sediment), different levels of contamination and duration of exposure (some years *vs* 20 days), and so this issue deserves further investigation.

Since the first organotin restrictions in the 1980's the increased use of copper in antifouling systems raised the concern of the impact of higher levels of this element in the environment due to higher metal quantities leached from vessels bottom paints (Voulvoulis *et al.*, 1999; Terlizzi *et al.*, 2001). Copper upward trends registered in some molluscs and fish in the European Atlantic coast may probably be attributable to the growing use of copper AFP subsequent to the organotin antifouling regulations adopted in the 1980's (OSPAR, 2007). However, there are also a number of examples in the European Atlantic coast where no obvious temporal trends occurred in the copper concentration in biota or even significant downward trends had occurred (OSPAR, 2007). In the current study we noticed no significant trends in the concentration of copper and TBT in the sediments and in the concentration of copper in the tissues of *N. reticulatus*; perhaps a larger temporal scale is needed to depict significant changes. In view of this, our data constitute an important baseline to investigate future temporal evolution of copper and TBT along the Portuguese coast. Presently, this topic deserves particular attention as organotins will be completely banned worldwide from September 2008 through the implementation of the "International Convention on the Control of Harmful Anti-fouling Systems on Ships" approved by IMO.

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CAPÍTULO 7

CHAPTER 7

Levantamento do Parasitismo por Trematódes Digenéticos em *Nassarius reticulatus* (L.) ao longo da Costa Portuguesa: Avaliação do Potencial Impacto na Reprodução e na Expressão do *Imposex*

Assessment of Digenean Parasitism in *Nassarius reticulatus* (L.) along the Portuguese Coast: Evaluation of Possible Impacts on Reproduction and Imposex Expression

Aceite para publicação na revista científica/Accepted for publication in:
Journal of Parasitology (www.ncbi.nlm.nih.gov/pubmed/18712950)

Abstract

A survey was performed between June and September 2006 along the Portuguese coast in order to assess the prevalence of digenean parasitism in the netted whelk *Nassarius reticulatus* - a bioindicator of tributyltin pollution. It was also intended to evaluate the effect of parasites on the reproduction of this gastropod species and their interference on the development of imposex (the superimposition of male characters onto prosobranch females) and male penis, based on field data. Five digenean species (*Lepocreadium album*, *Gynaecotyla longiintestinata*, *Himasthla quissetensis*, *Diphtherostomum brusinae*, and *Cardiocephalus longicollis*), plus one unidentified species, were found to infect *N. reticulatus*. Parasitism was spread along the Portuguese coast but the higher values of prevalence were found in sheltered inshore areas where up to 67.4% of the animals were affected per sampling station. Parasitism has a castrating impact on the whelks and a reducing effect on male penis size, which causes serious disorders in the reproduction of *N. reticulatus* and may have an important impact in the species population dynamics. No relation between imposex severity and parasite infestation was found.

Key words: *Nassarius reticulatus*, Trematodes, Imposex.

7.1 INTRODUCTION

Disruption of the endocrine system became, in the last decade, a subject for a wide variety of studies in the aquatic environment, both in vertebrate and invertebrate animals. Major causative agents of endocrine disruption are thought to be endocrine disruptor chemicals (EDCs), both natural and man-made. In invertebrates, the clearest example of chemically induced endocrine disruption is the masculinisation of female prosobranch gastropods when exposed to tributyltin (TBT) (Gibbs & Bryan, 1996), a biocidal compound mainly used in antifouling paints. Smith (1971) coined this phenomenon as "imposex", to describe "the superimposition of male characters onto unparasitized and parasitized prosobranch females". Imposex has been used as a biomarker of TBT pollution over the last decades in many studies throughout the world.

Endocrine disruption is not always caused by environmental pollutants and can occur as a consequence of other conditions and/or factors, including sub-optimal temperature, restricted food supply, low pH and parasites (Jobling & Tyler, 2003). Trematode parasites are ubiquitous organisms present in both marine and freshwater ecosystems. The adult digenean trematode parasite is resident in a vertebrate definitive host and sheds eggs into the aquatic environment. From the egg, a free-living larval stage (miracidium) emerges and infects a mollusc. Within the new first intermediate host, the parasite begins a series of asexual cycles, the complexity of which depends on species, which develop into cercariae, another larval stage, which emerge from the mollusc to the water. These free swimming larvae may infect another vertebrate or invertebrate, the second intermediate host, before transmission to the definitive (Esch *et al.*, 2001, Mouritsen & Poulin, 2002). In the mollusc, the trematode parasite has to adapt to the host's internal environment to obtain energy for its own metabolism and needs to avoid being attacked by the host's immune system. To meet their requirements, the parasites may interfere with the neuroendocrine regulation and the internal defence system of the host, resulting in a modulation of the host's defence and affecting its physiology (de Jong-Brink *et al.*, 2001), damaging their host to a certain degree. Damage to the host ranges from minor metabolic changes to severe modifications, such as allocation of secondary metabolites, behavioural changes, abnormal shell formation and tissue destruction (Jensen *et al.*, 2006; Morley *et al.*, 2006; Van den Broeck *et al.*, 2007). Digeneans frequently infect the gonadal tissues of the mollusc (Mouritsen & Bay, 2000) and this may cause castration (Oliva *et al.*, 1999; Probst and Kube, 1999; Tétreault *et al.*, 2000; Curtis, 2002) and may alter the development of sexual characters (Evans *et al.*, 2000; Tétreault *et al.*, 2000). It is obvious that digeneans have a major impact on the endocrine system of their molluscs' hosts and could hypothetically promote or restrain the imposex development in prosobranch gastropods, but their involvement in this phenomenon is not clear. Several studies have approached this question. Evans *et al.* (2000) investigated the potential of endocrine disruptors other than TBT that cause imposex in the dogwhelk *Nucella lapillus* (L.). They compared the occurrence of imposex and infestations of the trematode parasite *Parorchis acanthus* in *N. lapillus* collected in the Isle of Cumbrae, Scotland. They found that although parasites can affect the gonodal development in gastropods, there was no relation between the severity of imposex and the levels of parasitic infestation. Another study in the same mollusc-trematode system (Morley *et al.*, 2003) compared the intensity of imposex and the parasite prevalence in Northern Ireland and concluded that imposex severity was not affected by the prevalence of parasites. Curtis (1994) in a survey to determine the frequency, intensity and spatial distribution of imposex in *Ilyanassa obsoleta* from Cape Henlopen, Delaware, found only a weak correlation between the percentage of parasitized whelks and the percentage of imposex across sites, and only with two of the four trematode species studied.

The netted whelk *Nassarius reticulatus* is a gastropod recommended as bioindicator of TBT pollution (Joint Assessment and Monitoring Program guidelines; http://www.ospar.org) and has been used in several TBT monitoring programs along the Portuguese coast (Barroso *et al.*, 2000; Barroso *et al.*, 2002; Santos *et al.*, 2004; Barroso *et al.*, 2005; Sousa *et al.*, 2005; Rato *et al.*, 2006). This gastropod was also reported as first and second intermediate host of digenetic trematode parasites in Portugal (Russell-Pinto *et al.*, 2006). Despite the extensive imposex studies with this species in the area, no work has approached the potential relation between trematode parasitism and imposex development in *N. reticulatus*.

The aim of the present study is: (1) to assess the prevalence of parasitism in *N. reticulatus* (proportion of parasitized whelks in the population) along the Portuguese coast; (2) to identify the trematode species that infect the whelk in this geographical area; (3) to evaluate the effect of parasites in gastropod gonads and digestive gland; and (4) to determine if parasitism may interfere with the expression of the imposex in the females and the development of penis in the males.

7.2 MATERIALS AND METHODS

7.2.1 Sampling and pre-treatment

Nassarius reticulatus sampling was performed in 172 offshore and 48 inshore stations along the Portuguese coast (Figure 7.1) between June and September 2006. At each sampling station, the geographical co-ordinates were determined with a mobile global positioning system (GPS) (Table 7.1). The study area covers all the mainland coast of Portugal, including estuaries and the open seashore (for simplicity here designated as "inshore") and also the adjacent continental shelf off Aveiro, Lisbon and Setúbal (here designated as "offshore"). A number of studies have shown that the main TBT pollution sources in the Portuguese coast are the harbours (which comprise commercial and fishing ports, dockyards and marinas) (see Barroso *et al.*, 2002, and Sousa *et al.*, 2005) where a high number of ships and boats are anchored and thus an intense TBT leaching occurs from hull antifouling coatings (de Mora *et al.*, 1995; Stuer-Lauridsen & Dahl, 1995; Page *et al.*, 1996); for this reason the present survey includes sampling stations close to these hotspots and many others increasingly distant from these places in order to get gradients of imposex in the area. Specimens from inshore stations were collected by hand at intertidal sites or with baited hoop nets at sublitoral sites. Those from offshore stations were obtained by means of a 5 minutes tow using dredges on both sides of the boat. *N. reticulatus* specimens were maintained alive in aquaria and examined for imposex within 3 days after the collection. Adult animals (recognized by the presence of white columellar callus and teeth on lip of the shell) were selected and narcotized using 7% $MgCl_2$ in distilled water. Before shells were cracked open with a bench vice, their height (distance from shell apex to lip of siphonal canal) was measured with a vernier calliper to the nearest 0.1 mm. Then, individuals were sexed and dissected using a stereomicroscope, for the classification of imposex parameters and inspection of possible trematode parasite infections.

7.2.2 Imposex analysis

Three indices were determined for each sampling station to describe the intensity of imposex: the percentage of females affected by imposex (%I), the vas deferens sequence index (VDSI) and the female penis length index (FPLI). The VDSI was classified according to the scoring system proposed by Stroben *et al.* (1992), but the computation of the VDSI was based on the methodology proposed by Barroso *et al.* (2002), i.e., stages 4 and 4^+ were computed with the numerical values of 4 and 5, respectively, for a better discrimination of imposex levels between different sites. The male and female penis lengths were measured using a stereomicroscope, with a micrometric ocular. The FPLI was calculated as the average value of the penis length of females per sampling station. Unlike other imposex studies where animals presenting parasites are frequently discarded, in this work parasitized animals were also examined for the determination of the above imposex indices.

7.2.3 Trematode identification

Animals were carefully observed using a stereomicroscope in order to detect the possible presence of trematode parasites. In parasitized animals, a piece of infected tissue was taken from the digestive gland/gonad; squash slides were prepared (a drop of sea water was set on the slide and the piece of tissue placed on drop; tissue was gently pressed when placing the coverslip) and examined using light microscopy. The trematode identification was performed according to the morphological features described for trematode parasites in *N. reticulatus* (Russel-Pinto *et al.*, 2006).

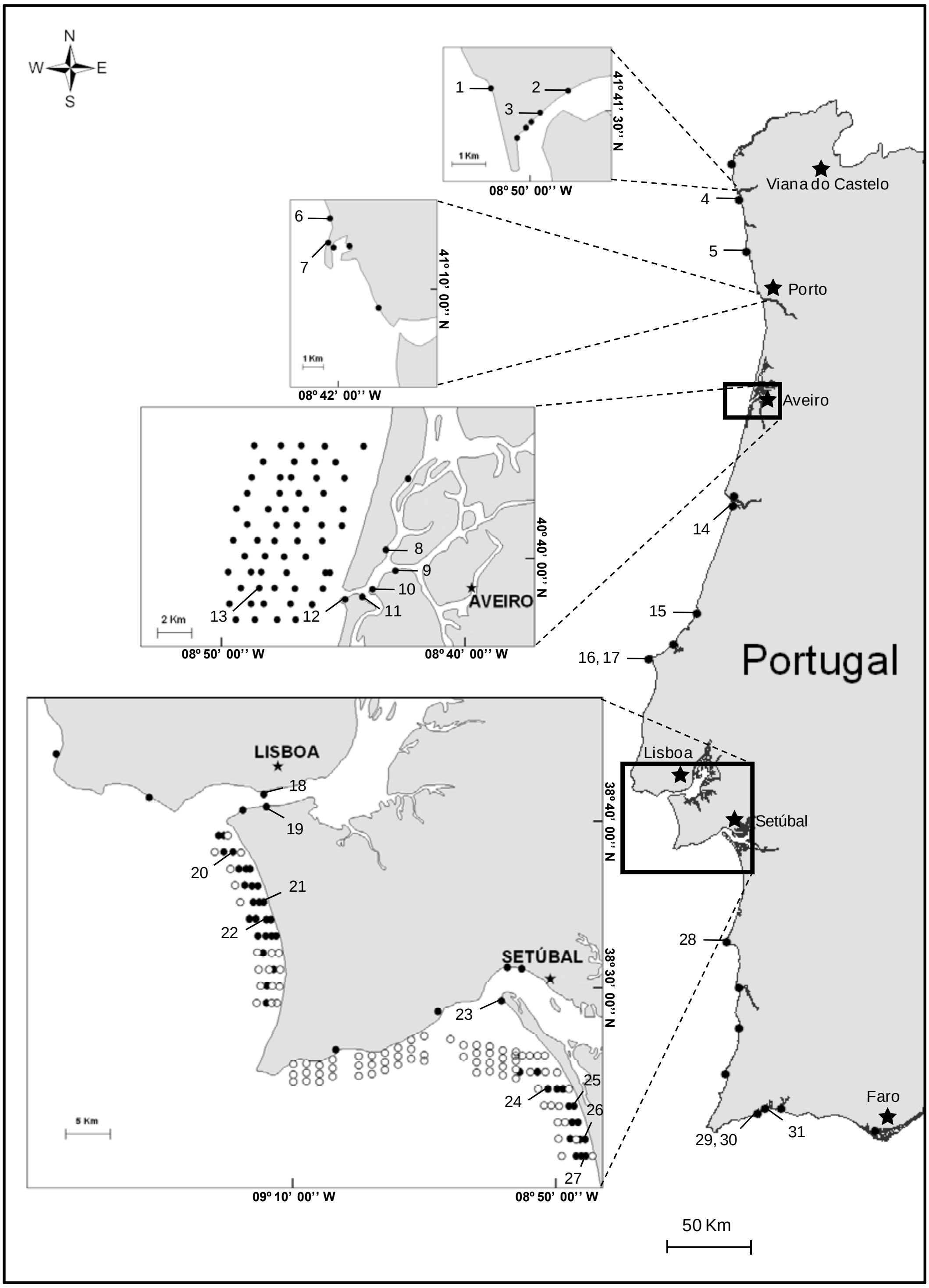

Figure 7.1 – *Nassarius reticulatus*. Map showing the study area and location of the sampling sites. Black circles represent sites where samples with 4 or more females were obtained and white circles represent sites where no females or a very low number of females (<4) were obtained and were not used in the analysis. The numbers correspond to sites where parasitized animals were found (Table 7.1).

7.2.4 Histological analysis

Parasitized animals were fixed in Bouin's solution for 24 – 48hr and then preserved in 70% ethanol. Later, the tissues were dehydrated by immersing specimens in successive alcohols with increasing concentrations, followed by immersion in a mixture of 100% ethanol + benzene, and finally in pure benzene. After dehydration, the specimens were embedded in paraffin and serially cut in sections of 7 – 10 µm. Each three to four longitudinal sections were placed in slides and then deparaffinized with xylene. Tissues were rehydrated with successive decreasing alcohol concentrations (ethanol 100º, ethanol 90º, ethanol 75º, ethanol 50º), stained with haematoxylin and eosin, once again dehydrated (ethanol 95º, ethanol 100º) and cleared with xylene. Slides were mounted with a drop of mounting medium for microscope preparations and examined by light microscopy.

7.2.5 Data analysis

Several statistical analyses were applied to data using the SigmaStat (Systat Software, Inc., San Jose, California). The proportion of parasitized and unparasitized males and females was analyzed with a Fisher's exact test. The non-parametric Spearman's rank coefficient was used for the correlations analysis. The differences between two unpaired groups were assessed using Mann-Whitney *U*-tests. Simultaneous testing and modelling of multiple independent variables were evaluated by Ordinary Least Squares (OLS) linear regression, after checking that normal distribution and the constant variance of the errors were not violated. The analysis of variance between groups was performed using ANOVA and if the ANOVA assumptions (normality and homogeneity of variances) were not met, non-parametric tests were run and are indicated in the text.

7.3 RESULTS

7.3.1 Taxonomic identification of trematodes and prevalence of parasitism

Nassarius reticulatus was found in almost all inshore stations sampled, 45 of a total of 48 sites visited, but only in 97 of a total of 172 offshore stations. Parasitized specimens occurred in about half (51.1%) of the inshore stations, but only in 8.2% of the offshore stations where whelks were collected. The prevalence of parasitized animals per sampling station varied between 0 – 14.3% and 0 – 67.4% in offshore and inshore areas, respectively, affecting equally both genders (Fisher's exact test, P=1.000). The stations where parasites were found are described in Table 7.1. In this study only single infections were observed, i.e., only one parasite species was found inside a whelk, based on the cercariae identification on squash slides. Parasite identification and respective prevalence are presented in Table 7.2. A total of six species were detected in *N. reticulatus*. All these species were described by Russel-Pinto *et al.* (2006) for this gastropod in the Ria Aveiro (Portugal), including a zoogonidae cercarium of an unidentified species (this larval stage could not yet be associated to any species described in the literature). The cercaria of *Lepocreadium album* (Lepocreadiidae; Nicoll, 1934) was the most abundant trematode, accounting for 58.8% of the observations, followed by the zoogonid cercaria (15.3%), cercaria of *Gynaecotyla longiintestinata* (Microphallidae; Leonov 1958) (10.7%), *Himasthla quissetensis* (Echinostomatidae; Looss 1902) (7.6%), *Diphtherostomum brusinae* (Zoogonidae; Odher 1911) (4.6%) and the less abundant *Cardiocephalus longicollis* (Strigeidae; Raillet 1979) (3.0%).

Table 7.1 – *Nassarius reticulatus*. Data relative to stations where parasitized animals were found, with indication of the station code, station name, coordinates (latitude and longitude), proximity to TBT source (C – close; N – near; F – Far), type of sediment (S – sandy; M – muddy), location of sampling site (O – offshore; CL – coastal line; IE – inside estuary), number of females (N ♀), number of males (N ♂), imposex incidence (%I), vas deferens sequence index (VDSI), female penis length index (FPLI), percentage of parasitized females (%♀), percentage of parasitized males (% ♂), and total prevalence of parasitized animals in the sample (%P).

Station Code	Station Name	Lat (N)	Long (W)	Proximity	Sediment	Location	N ♀	N ♂	%I	VDSI	FPLI	%♀	% ♂	%P
1	Praia Norte	41º 41.85	08º 51.13	N	S	CL	54	34	90.7	1.35	0.08	1.8	0.0	1.1
2	Viana Castelo - Marina	41º 41.70	08º 49.20	C	M	IE	31	36	96.8	2.74	1.15	6.4	8.1	7.4
3	Viana Castelo - Marégrafo	41º 41.43	08º 49.71	C	S	IE	47	39	100.0	3.26	1.82	10.6	5.7	8.5
4	Praia da Amorosa	41º 38.72	08º 49.31	N	S	CL	63	12	82.5	1.41	0.12	1.6	0.0	1.0
5	Póvoa de Varzim	41º 23.18	08º 46.40	N	S	CL	62	36	46.8	0.59	0.03	1.6	0.0	1.0
6	Praia de Leça	41º 12.21	08º 42.82	N	S	CL	8	43	50.0	0.75	0.03	0.0	2.3	2.0
7	Porto de Leixões- Marina	41º 11.30	08º 42.24	C	M	IE	40	45	100.0	4.15	6.38	2.5	0.0	1.2
8	Aveiro - S. Jacinto	40º 39.48	08º 43.56	C	M	IE	38	22	97.4	2.42	0.99	34.2	31.8	33.3
9	Aveiro - Terminal Químico	40º 39.46	08º 42.74	C	M	IE	32	27	100.0	3.13	1.45	3.7	3.6	2.1
10	Aveiro - PCN	40º 39.06	08º 43.76	C	M	IE	41	22	100.0	2.32	3.83	0.0	4.6	1.6
11	Aveiro - Maglhães Mira	40º 38.65	08º 44.06	C	M	IE	40	20	100.0	2.85	1.09	10.0	10.0	10.0
12	Aveiro - Barra	40º 38.71	08º 44.82	C	S	IE	36	23	88.9	2.83	1.12	2.8	0.0	1.7
13	Offshore	40º 39.00	08º 48.37	F	S	O	22	19	40.9	0.82	0.26	4.6	0.0	2.4
14	Figueira Foz - Estaleiro	40º 08.60	08º 51.55	C	M	IE	24	31	79.2	1.29	0.14	41.7	38.7	40.0
15	Nazaré - Porto de Pesca	39º 35.04	09º 04.39	C	M	IE	10	36	100.0	4.10	5.74	50.0	72.2	67.4
16	Peniche - ISN	39º 21.20	09º 22.70	C	M	IE	8	26	100.0	4.62	4.15	0.0	42.3	32.4
17	Peniche - Porto de Recreio	39º 21.15	09º 22.52	C	M	IE	8	51	100.0	4.50	4.41	0.0	2.0	1.7
18	Lisboa - Marina de Belém	38º 41.50	09º 12.50	C	M	IE	22	29	100.0	3.36	4.45	0.0	12.5	8.6
19	Lisboa - Porto Brandão	38º 40.77	09º 12.29	C	M	IE	33	27	100.0	3.67	2.72	3.0	0.0	1.7
20	Offshore	38º 38.00	09º 14.55	F	S	O	6	10	83.3	1.17	1.07	16.7	0.0	6.2
21	Offshore	38º 35.00	08º 12.38	F	S	O	18	16	83.3	0.89	0.03	5.6	0.0	3.0
22	Offshore	38º 34.00	08º 12.13	F	S	O	30	42	80.0	0.93	0.27	3.3	0.0	1.4
23	Setúbal - Tróia	38º 29.40	08º 54.14	C	S	IE	26	31	100.0	4.15	4.50	0.0	3.2	1.8
24	Offshore	38º 24.00	08º 49.91	F	S	O	14	7	7.1	0.08	0.00	7.4	0.0	5.0
25	Offshore	38º 23.00	08º 48.79	F	S	O	21	8	14.3	0.14	0.00	4.8	12.5	6.9
26	Offshore	38º 21.00	08º 47.93	F	S	O	22	14	18.2	0.18	0.00	4.6	0.0	2.8
27	Offshore	38º 20.00	08º 47.30	F	S	O	4	3	0.0	0.00	0.00	25.0	0.0	14.3
28	Sines - Porto de Pesca	37º 57.28	08º 52.21	C	M	IE	17	57	100.0	4.41	4.62	0.0	5.6	4.2
29	Lagos - Porto de Pesca	37º 06.30	08º 40.33	C	M	IE	24	14	100.0	3.83	1.88	0.0	7.1	2.6
30	Lagos - Barra	37º 06.08	08º 40.15	C	M	IE	37	22	100.0	3.43	2.10	2.7	4.6	3.4
31	Alvor - Barra	37º 07.22	08º 37.14	N	S	IE	31	29	38.7	0.39	0.01	3.2	6.9	5.0

Table 7.2 – *Nassarius reticulatus*. Data relative to parasite species found with indication of percentage of parasitized females (%♀ relative to total number of females in the sample) and percentage of parasitized males (%♂ relative to total number of males in the sample) for the 6 digenean, *Lepocreadium album*, *Gynaecotyla longiintestinata*, *Diphtherostomum brusinae*, *Cardiocephalus longicollis*, *Himasthla quissetensis* and unidentified species (Zoogonidae cercariae). In offshore stations no squash slides were performed from the parasitized whelks and consequently the occurring trematode species were not observed for identification (NOI).

Station Code	Trematode Species	%♀	%♂
1	Zoogonid cercariae	1.85	
2	*L. album*	3.23	8.57
	Zoogonid cercariae	3.23	
3	*L. album*	6.38	
	Zoogonid cercariae	4.26	5.13
4	Zoogonid cercariae	1.59	
5	Zoogonid cercariae	1.61	
6	Zoogonid cercariae		2.33
7	*D. brusinae*	2.50	
8	*L. album*	2.63	8.70
	G. longiintestinata	26.32	4.35
	D. brusinae	2.63	4.35
	C. longicollis	2.63	
	Zoogonid cercariae		13.64
9	*D. brusinae*		3.70
	Zoogonid cercariae	3.13	
10	Zoogonid cercariae		4.55
11	*D. brusinae*	2.50	
	C. longicollis	2.50	
	H. quissetensis	2.50	
	Zoogonid cercariae	2.50	10.00
12	*C. longicollis*	4.35	
13	NOI	4.55	
14	*L. album*	33.33	35.48
	Zoogonid cercariae	8.33	3.23
15	*L. album*	50.00	72.22
	Zoogonid cercariae	8.33	3.23
16	*L. album*		7.69
	H. quissetensis		34.62
17	*L. album*		1.96
18	*L. album*		9.26
	D. brusinae		1.85
19	Zoogonid cercariae	3.03	
20	NOI	16.67	
21	NOI	5.56	
22	NOI	3.33	
23	*L. album*		3.23
24	NOI	7.14	
25	NOI	4.76	12.50
26	NOI	4.55	
27	NOI	25.00	
28	*G. longiintestinata*		5.26
29	*L. album*		7.14
30	*L. album*	2.70	4.55
31	*L. album*	3.23	3.45
	C. longicollis		3.45

7.3.2 Histological analysis

Histological analyses were performed on thirty specimens. Some of these specimens exhibited a single infection by the following digenean species: *L. album*, *G. longiintestinata*, *H. quissetensis* and the zoogonid cercariae – an unidentified species. Other specimens were unparasitized. The slide examinations revealed that the larval parasitic stages infected both the digestive gland and the gonad of the whelk (Figure 7.2). In the digestive gland, the sporocysts were found within the tubules, in some cases compressing the surrounding tissue, whereas affected gonads showed a severe histological disorganization. In most cases, the gonadal tissue of *N. reticulatus* was completely replaced by the larval stages of the parasites.

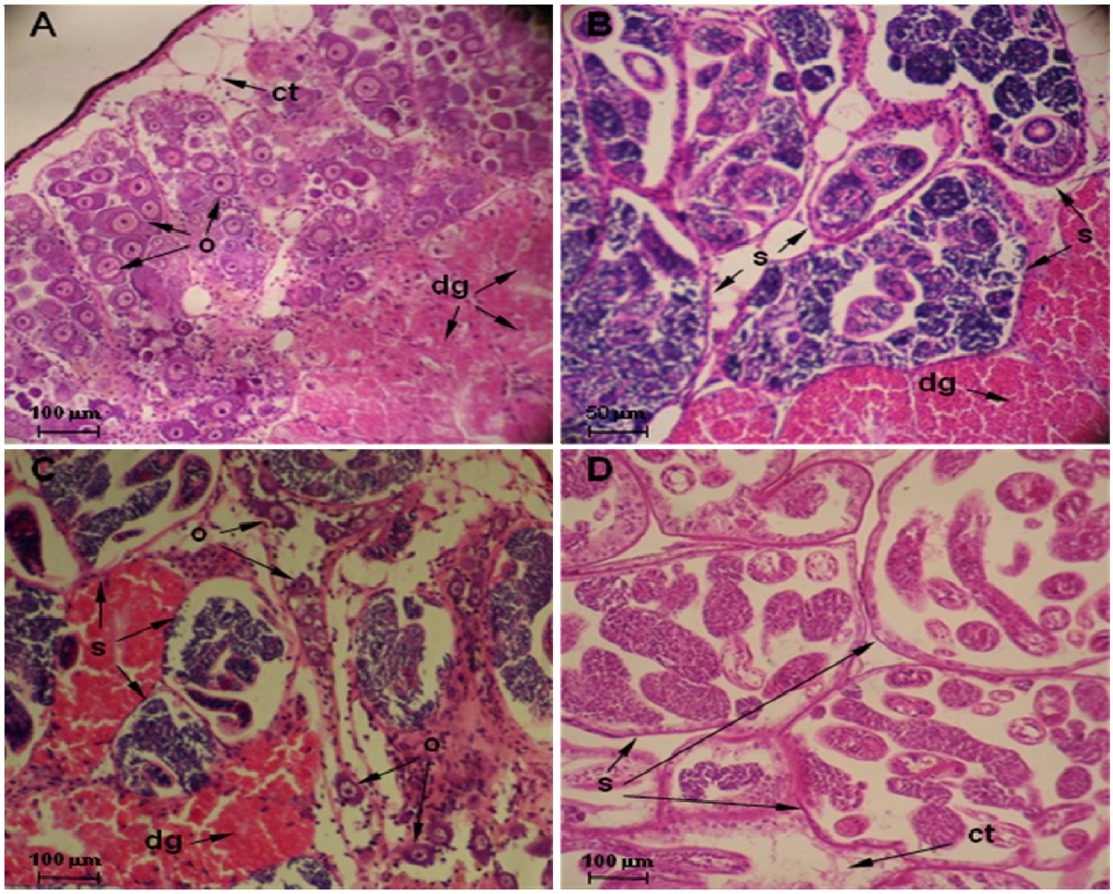

Figure 7.2 – *Nassarius reticulatus*. Histological sections showing (**A**) unparasitized female, (**B**) female infected by the zoogonid cercariae (see text), (**C**) female infected by *G. longiintestinata*, (**D**) male infected by *L. album*; ct – connective tissue, dg – digestive gland, o – oocyte, s – sporocyst.

7.3.3 Influence of trematode parasitism on imposex

A. Correlations between parasite prevalences and %I, VDSI and FPLI

Data in Table 7.1 indicate that there is a wide variety of imposex intensities across the 31 sites where parasitism occurs. At the offshore sites where *N. reticulatus* is affected with parasitism, %I ranged between 0 and 83.3%, VDSI varied between 0 and 1.17 and FPLI ranged between 0 and 1.07. Similarly, in the inshore sites where parasitism occurred, %I, VDSI and FPLI varied, respectively, between 38.7 – 100%, 0.39 – 4.62 and 0.01 – 6.38 (Table 7.1). Furthermore, the prevalence of parasitized females across these sites shows no obvious trend with the level of imposex (Figure 7.3) as no significant correlation was observed between the prevalence of parasitized females (%PF) and the imposex indices %I (r=-0.07, P=0.750), VDSI (r=-0.18, P=0.592) and FPLI (r=-0.03, P=0.890).

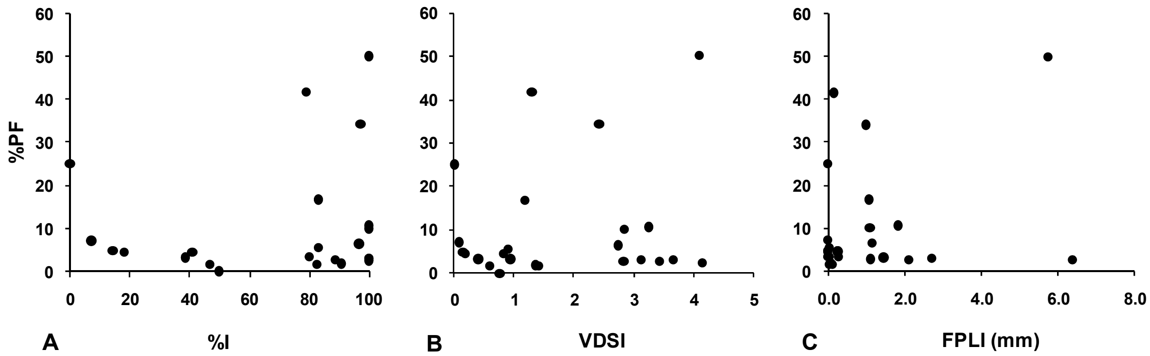

Figure 7.3 – *Nassarius reticulatus*. Relationship between prevalence of parasitized females (%PF) and (**A**) imposex incidence (%I), (**B**) vas deferens sequence index (VDSI) and (**C**) female penis length index (FPLI).

These results suggest that there is no apparent relationship between parasitism and imposex in *N. reticulatus* at these sites. However, when field data are used to address the question "may the parasitism influence the expression of imposex?" one should have in mind that mixed factors could be involved in the expression of imposex and that they should be included in the analyses. An obvious factor is TBT as it is known that the imposex intensity depends on the degree of *N. reticulatus* TBT tissue contamination at each site. Moreover, when one refers to FPLI, this imposex index may depend on TBT and also on animal's size. In the following paragraphs, the possible influence of each of these factors and parasitism on imposex levels of *N. reticulatus* are compared.

B. VDS vs parasitism and TBT

In the current survey, there are no available data regarding the TBT pollution at each site, but the sources of TBT pollution in the Portuguese coast are very specific and localized (ports, dockyards and marinas) and pollution decreases with the distance from these sources (Barroso *et al.*, 2002; Sousa *et al.*, 2005; Rato *et al.*, 2006; Rato *et al.*, 2008). Thus, using the distance from the source as a proxy to estimate the level of TBT pollution at each site, a two factorial ANOVA Holm-Sidak test was performed to analyze the variation of VDS with the proximity of TBT sources (three categories were identified: close - $\leq$ 0.8 km, near - between 0.8 and 5.5 km, and far - $\geq$ 5.5 km from TBT sources) and with the presence/absence (two categories) of parasitism. Only the 23 sampling stations where parasitized females occurred were considered for this analysis and each female corresponded to an observation (N=756). The results showed that there was a significant effect of the proximity of TBT sources on the VDS level of each female (F=82.76, P<0.001), but there was no significant effect of parasitism (F=1.05, P=0.305).

A further step in the analysis consisted of examining the sampling sites separately, and comparing imposex between parasitized and unparasitized females within each site. In this way, it is possible to eliminate the spatial variability of TBT pollution and other possible local factors that may influence imposex. The difference between VDS values of parasitized and unparasitized females was assessed in stations where each of these two groups was represented by $\geq$4 females (stations 3, 8, 11, 14 and 15), otherwise the tests could fail due to lack of observations. No significant differences were found between the two groups in any of these stations, using the non-parametric Mann Whitney *U*-test: at station 3: U=102.00, P=0.928, number of unparasitized females (N_{unpar})=42, number of parasitized females (N_{par})=5; at station 8: U=113.50, P=0.450, N_{unpar}=25, N_{par}=13; at station 11: U=88.00, P=0.427, N_{unpar}=36, N_{par}=4; at station 14: U=73.50, P=0.846, N_{unpar}=14, N_{par}=10; at station 15: U=21.00, P=0.095, N_{unpar}=5, N_{par}=5.

C. FPL vs parasitism, TBT and shell height

To study the potential factors influencing the penis development in females, an Ordinary Least Squares (OLS) multiple linear regression analysis was carried out comparing the penis length of each female (dependent variable), and the respective shell height, the proximity of the site where the female was collected to TBT sources (three categories as above) and if the female was parasitized or unparasitized. After evaluating the variables significance and the adjusted R^2 the only covariate that proved to have a significant effect over the FPL was the proximity to TBT sources (OLS general fit: R^2=0.21, F=76.98, P<0.001; Shell height: beta=-0.05, t=-1.67, P=0.096; Proximity to source: beta=-0.45, t=-14.19, P<0.001; Parasitism: beta=-0.01, t=-0.38, P=0.707).

The influence of parasitism on the female penis length was also analyzed per station. The data from the above five stations, used in VDS analysis, were tested to assess the difference between the penis length of parasitized and unparasitized females and again no significant differences were found (station 3: U=74.50, P=0.300; station 8: U=113.50, P=0.450; station 11: U=96.50, P=0.276; station 14: U=71.00, P=0.073; station 15: U=21.50, P=0.095).

7.3.4 Influence of trematode parasitism on male penis length

The impact of parasites on the male penis development was also evaluated and the results showed that in almost all the stations parasitized males have smaller penis (Figure 7.4). An OLS linear regression analysis was carried out between the penis length of each male (dependent variable) and the respective shell height and if the male was parasitized or unparasitized. Only stations where parasitized males occurred were considered for this analysis, each male corresponding to an observation (N=543). Both covariates proved to have a significant effect over the male penis length (Table 7.3). Nevertheless, only around 15% of the variance was explained by this regression and this is expected since it is known that penis length is influence by local parameters. In fact, the male penis length depends on the sexual maturation stage (Barroso & Moreira, 1998), which may change from site to site and was not assessed in this work. Besides, male penis length may eventually also depend on animal’s condition and other environmental factors. To avoid variation related to local parameters, an analysis was performed considering each station individually (Figure 7.4).

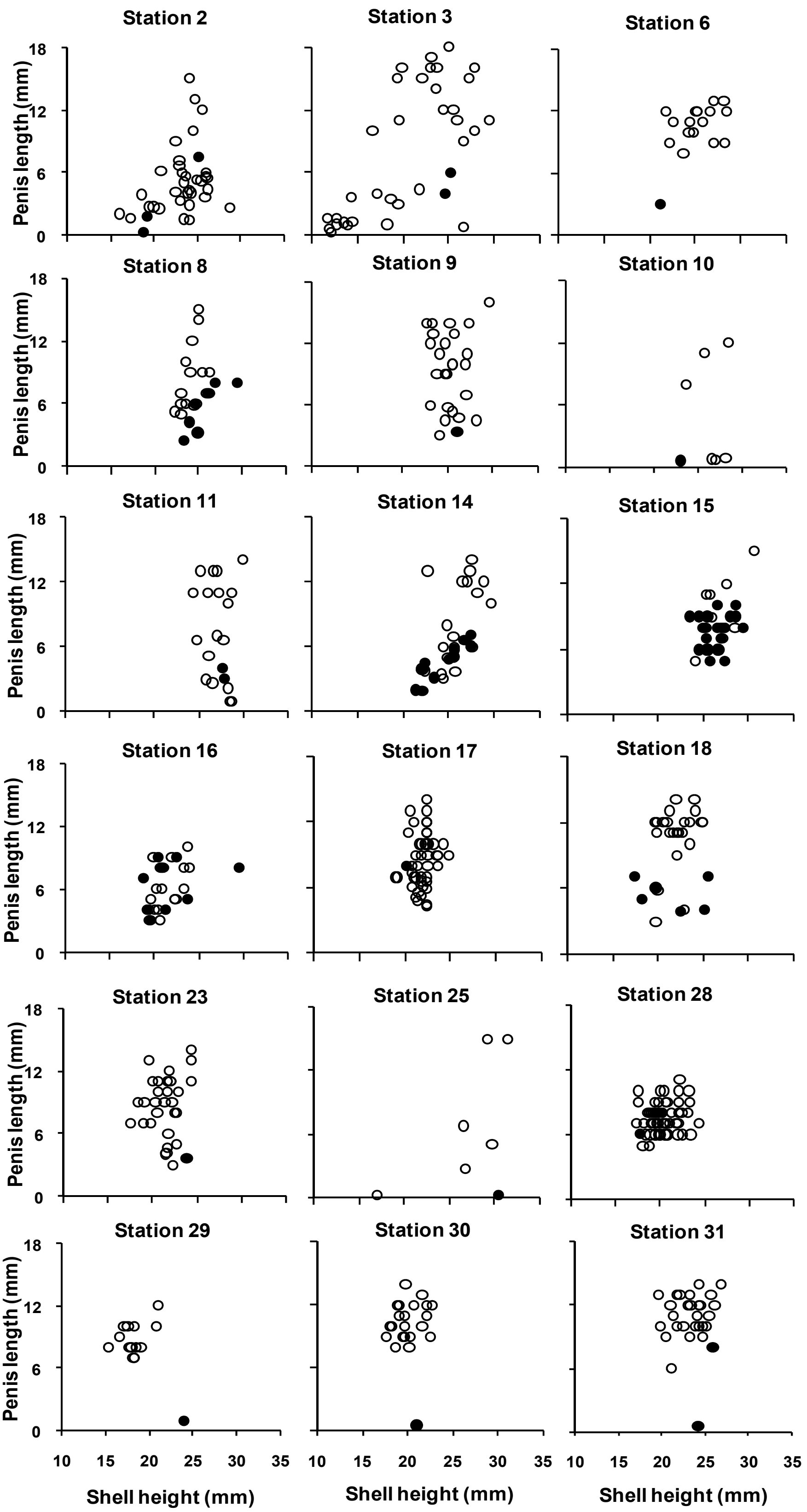

Figure 7.4 – *Nassarius reticulatus*. Relation between male penis length and shell height of parasitized (●) and unparasitized (○) animals. For OLS regression compare with Table 7.3.

Table 7.3 – OLS regression parameters for analysis of the relationship between male penis length versus shell height and parasitism, indicating the statistical parameters for the general fit: the number of unparasitized males (N_{unpar}), the number of parasitized males (N_{par}), the coefficient of determination (R^2), the F-test value (F) and the respective significance (P), and statistical beta parameters for both shell height and parasites: the beta coefficients (Beta), the t-test values (t) and the significance (P). "All stations" refers to 18 sampling stations where parasitized males were observed.

Station Code	N_{unpar}	N_{par}	General fit			Shell height beta coefficient			Parasites beta coefficient		
			R^2	*F*	*P*	Beta	*t*	*P*	Beta	*t*	*P*
All stations	461	82	0.15	41.69	<0.001	0.25	5.96	<0.001	-0.34	-7.89	<0.001
8	15	7	0.40	4.46	<0.001	0.52	2.67	<0.05	-0.64	-3.29	<0.001
14	19	12	0.50	13.85	<0.001	0.54	3.93	<0.001	-0.35	-2.52	<0.05
15	10	26	0.22	4.75	<0.05	0.37	2.38	<0.05	-0.26	-1.66	0.107
16	15	11	0.14	1.81	0.186	0.36	1.83	0.080	0.11	0.56	0.578
18	23	6	0.43	9.77	<0.001	0.13	0.85	0.406	-0.63	-4.22	<0.001

Hence, for those stations where the number of observations for parasitized and unparasitized males is sufficient (≥4) (stations 8, 14, 15, 16 and 18), OLS linear regression analyses were performed. The regression parameters for these stations are given in Table 7.3 showing in some cases that one, or both, covariates had a significant effect on penis length. Globally, this statistical analysis and the inspection of Figure 7.4 show that penis tends to be longer for increasing shell heights and smaller in parasitized animals.

7.4 DISCUSSION

Nassarius reticulatus is an ubiquitous gastropod of European coasts that has been used by several authors (Stroben *et al.*, 1992; Bryan *et al.*, 1993; Barreiro *et al.*, 2001; Santos *et al.*, 2004; Barroso *et al.*, 2005; Ruiz *et al.*, 2005; Sousa *et al.*, 2005; Rato *et al.*, 2006) in studies of imposex assessment, an induced endocrine disruption phenomenon caused by organotin compounds of antifouling paints (mainly TBT). In this work, *N. reticulatus* was found to be an intermediate host for six species of digenean trematode parasites, *Cardiocephalus longicollis*, *Cercaria sevillana* (the cercariae of *Gynaetotyla longiintestinata*), *Diphtherostomum brusinae*, *Himasthla quissetensis*, *Lepocreadium* *album* and one unknown zoogonid cercariae. These species were previously described by Russell-Pinto *et al.* (2006) for *N. reticulatus* in the Ria de Aveiro (Portugal).

In the present study, the parasite prevalence varied between 0 and 67.4% and parasites were found to infect specimens from different habitat types (offshore/inshore, inside estuaries/outside estuaries), showing that parasitism has a wide spatial distribution along the Portuguese coast. The highest levels of parasitism (>32% of parasite prevalence) were found in sheltered inshore stations located in enclosed body waters (stations 8, 14, 15 and 16). These higher values may be explained mainly by the expected higher occurrence of the definitive hosts in those areas, an essential factor for trematodes parasites to complete their life cycles. In the case of the five known digenean species, the definitive hosts are seabirds for *C. longicollis* (Prevot and Bartoli, 1980), *G. longiintestinata* and *H. quissetensis* (Stunkard, 1938) and fishes for *D. brusinae* (Williams and Jones, 1994) and *L. album* (Bray *et al.*, 1993). Sheltered areas enclose breeding colonies and roosting grounds and also offer food sources (either natural or organic wastes derived from human activities) allowing the congregation of high number of vertebrates (birds and fishes) and invertebrates (molluscs and crustaceans) that act, respectively, as definitive and intermediate hosts. Sexual reproduction of trematodes parasites takes place inside the gastrointestinal tract of the definitive host, and trematode eggs are passed out into the external environment with the faeces of the host. Gastropods subsequently become infected when they are penetrated by miracidia hatched from trematode eggs (Fredensborg *et al.*, 2006). The proximity of the definitive host increases the probability of the gastropod to get infected by trematodes parasites (Poulin & Mouritsen, 2003). Bustnes & Galaktionov (1999) refer to higher prevalence of trematodes in *Littorina saxatallis* within sheltered areas where seagulls are more abundant. Another study, performed by Fredensborg *et al.* (2006), clearly demonstrates a positive relationship between seabird abundance and trematode prevalence in the intertidal snail *Zeacumantus subcarinatus*.

The impact of trematode on their molluscan intermediate host has been described by several studies (Jensen *et al.*, 2006; Morley *et al.*, 2006; Van den Broeck *et al.*, 2007) and, in most of the whelk-trematode systems, the growth and reproduction of trematodes occur in the digestive gland/gonad complex of the gastropod (Probst & Kube, 1999). A possible consequence of parasitic infections is the castration of the host, which is the partial or total inhibition of gametogenesis in the host species due to the activity or physical presence of the parasite (Tétreault *et al.*, 2000). The mechanisms by which parasites castrate gastropods include the interference with the hormonal production and physical destruction of the reproductive tissues of the host (Fredensborg *et al.*, 2005).

In this work, the tissue alterations caused by four trematode parasite species were examined through histological studies. The larval stages were found in both digestive gland and gonad of *N. reticulatus*. In the affected gonads, the reproductive tissue had been replaced by parasite asexual stages, revealing that these trematode species have a castration effect upon *N. reticulatus*. Consequently, alterations in the gastropod endocrine physiology are expected to occur and could hypothetically influence the imposex expression. Few studies have approached the relationship between trematode infection and imposex and none has addressed the species *N. reticulatus*. In the present study, both parasitized and unparasitized females were found to have a wide range of imposex levels and no relation was found between trematode parasites prevalence and either of the imposex indices, %I, VDSI or FPLI, across sites. The differences of VDS values were tested in relation to the proximity to TBT sources and parasitism status. As expected, since imposex intensity depends on the TBT contamination at each site, VDS values were found to differ in relation to proximity to TBT sources, but no differences were observed related to parasitism. However, to avoid the spatial variability of TBT pollution and other possible local influencing factors, individual station analysis was performed. No difference was found between the mean values of VDS from parasitized and unparasitized females within each station, which supports the previous findings. The same conclusion was obtained for the female penis development, i.e., it depends on the proximity of TBT sources, but we could not prove that it is influenced by parasitism. When analyzed per station, no differences were found in the penis length values between parasitized and unparasitized females. The apparent lack of relation between the imposex severity and the parasitism infestation was also reported by Evans *et al.* (2000) and Morley *et al.* (2003), in studies where the *Nucella lapillus-Parorchis acanthus* system was investigated. Similarly, Curtis & Barse (1990) reported that the occurrences of parasitism and imposex in *I. obsoleta* appear to be independent of one another.

The relative penis length index (RPLI), which is defined as mean female penis length x 100/mean male penis length, is another imposex parameter frequently used in TBT pollution monitoring studies when *N. reticulatus* is used as bioindicator. As pointed out by the definition, this index relies not only on the female penis length, but also on the male penis length and therefore this index may be influenced by any changes in the male penis size. In a first approach, the results of this work reveal that the male penis length is reduced by the presence of trematode parasites. Evans *et al.* (2000), in the study on the relationship between the occurrence of imposex and infestations of parasitic trematode larvae, refer to studies of Køie (1969, 1975) who reported that, besides castration, trematode infections also cause degeneration of the male penis in *Buccinum undatum* and *Nassarius pygmaeus*. This reducing effect on penis size of gastropod males was also reported by Tétreault *et al.* (2000) and Morley (2006). It is important to note that contrary to mature infections which are easily identified, immature infections are more difficult to be detected. However, even early stages of parasitism can influence the development of the host's reproductive system (de Jong-Brink *et al.*, 2001), and we cannot reject the possibility that we have missed parasitized animals at these stages of infection. Nevertheless this is a contingency, especially in TBT pollution monitoring surveys that need to be performed in short periods of time. At least the identified parasitized animals should be discarded from imposex monitoring studies to minimize the influence of other factors than TBT on the values of the imposex indices based on the male penis length.

One ecological relevant aspects of the current study refers to the impact of trematode parasitism in the gastropod populations. These parasites have a major influence on the reproduction of the species as they cause castration in both genders and reduce the penis length in males, impairing the chances of breeding. This is particularly important at sites with high prevalence of parasitism, like for example, stations 8, 14, 15 and 16 where the prevalence is >32% and attains a maximum value of 67% at station 15 (Porto de Nazaré).

In conclusion, despite the serious disorders caused by trematode parasites on the reproductive system of *N. reticulatus*, which may have an important impact in the species population dynamics, it seems that parasitism has no influence in the expression of imposex in this gastropod. Nevertheless, as these results were originated from field data, further studies should be carried out under controlled conditions in the laboratory.

Acknowledgement

This work was developed under the research project POCI/MAR/61893/2004 financed by the FCT and by the POCI 2010, co-financed by FEDER. This work was supported trough a PhD grant (SFRH/BD/12441/2003) attributed by the Portuguese Foundation for Science and Technology (FCT).

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CAPÍTULO 8

CHAPTER 8

Conclusão Geral

General Conclusion

8.1 Evolução espacial da poluição por TBT na costa portuguesa

No presente trabalho *Nassarius reticulatus* foi utilizado como bioindicador para descrever a variação espacial e temporal da poluição por tributilestanho (TBT) na costa portuguesa. Uma das razões para a escolha desta espécie deve-se ao facto deste gastrópode prosobrânquio constar da lista de espécies recomendadas pela OSPAR (OSPAR, 2003) para programas de monitorização da poluição por TBT na região Nordeste do Atlântico, onde se inclui Portugal. Outra razão relaciona-se com o facto de estudos anteriores terem demonstrado que *N. reticulatus* é abundante na costa portuguesa e apresenta uma sensibilidade adequada para descrever os níveis de poluição existentes.

Neste estudo, confirmou-se que *N. reticulatus* era bastante comum ao longo da linha de costa portuguesa, apesar do baixo número ou mesmo ausência de animais em algumas das estações de amostragem visitadas, particularmente na costa sul. No entanto, as estações em que a espécie foi colhida providenciaram uma boa cobertura da área costeira de Portugal, uma vez que foram encontrados espécimes em torno dos principais portos nacionais (Viana de Castelo, Leixões, Aveiro, Figueira da Foz, Lisboa, Setúbal, Sines, Portimão e Faro) e nas zonas costeiras adjacentes. Contudo, *N. reticulatus* não foi o único nassarídeo amostrado durante o estudo, tendo sido colhidos também espécimes de *N. pygmaeus*, *N. nitidus* e *N. incrassatus*, mas em muito menor abundância. De entre estas espécies, *N. incrassatus* também foi proposta como espécie indicadora por Oehlmann *et al.* (1998) para a monitorização da poluição por TBT. Neste trabalho verificou-se, porém, que esta espécie é menos sensível do que *N. reticulatus* para avaliação dos actuais níveis de poluição por TBT, o que aliado à sua menor abundância, faz com que esta espécie seja menos adequada para este tipo de estudos nesta área. Outras espécies, também recomendadas na literatura como bioindicadoras da poluição por TBT, tais como *Hydrobia ulvae*, *Littorina littorea*, *Nucella lapillus*, *Ocenebra erinacea* e *Ocinebrina aciculata*, foram encontradas e colhidas nas campanhas decorridas ao longo da costa mas não foram objecto deste estudo. No entanto, será relevante mencionar que *N. reticulatus* foi, no geral, a espécie mais abundante ao longo da linha de costa e no interior de estuários, e a única espécie bioindicadora existente na plataforma continental adjacente, pelo que poderá ser considerada, em termos de monitorização à escala nacional, como o principal bioindicador da poluição por TBT.

Os níveis de *imposex* de *N. reticulatus* mais elevados foram encontrados nas estações situadas dentro ou na proximidade de zonas portuárias. Este padrão espacial também foi observado em estudos anteriores para a costa ocidental portuguesa (Barroso *et al.*, 2002a; Sousa *et al.*, 2005) e estes dados vêm reforçar o conceito de que locais que apresentam uma intensa actividade naval, tal como portos, marinas e estaleiros, são as principais fontes de poluição por TBT. Isto acontece porque nestes locais ocorre uma intensa lixiviação do TBT a partir das tintas antivegetativas das embarcações para a água e, onde existem estaleiros navais, pode haver escorrência de águas contaminadas e com partículas de tinta directamente para o meio marinho/estuarino envolvente, resultantes da decapagem dos cascos dos navios (Evans *et al.*, 1995; Bech, 2002; Gibson and Wilson, 2003; Harino *et al.*, 2005).

Os resultados obtidos neste trabalho demonstraram que a poluição por TBT é elevada em quase todas as estações analisadas ao longo da linha de costa de Portugal. Na realidade, e dado que a monitorização dos efeitos específicos do TBT se tornou mandatária sob a jurisdição do CEMP ("Coordinated Environmental Monitoring Program"), a OSPAR adoptou os "Provisional JAMP Assessment Criteria for TBT-specific biological effects" (OSPAR, 2004) de forma a extrapolar, a partir dos valores de VDSI de algumas espécies de gastrópodes bioindicadoras – onde se inclui *N. reticulatus* – quais os locais onde os níveis de contaminação ambiental por TBT são superiores ao EAC ("Environmental Assessment Criteria"). No caso da água os valores de EAC são 0,004 – 0,04 ng TBT-Sn/L e no sedimento são 0,002 – 0,02 ng TBT-Sn/g (peso seco) (OSPAR, 1997). No caso de *N. reticulatus* considera-se que os valores de EAC num determinado local são ultrapassados quando o VDSI desta espécie para esse local é superior a 0,3 (OSPAR, 2004). Só em dois locais da linha de costa portuguesa, dos quarenta e quatro locais amostrados, se registaram valores de VDSI inferiores a 0,3, correspondendo à classe A-B. Todas as outras estações caem nas classes C a E (ver 1.4.4), indicando que, nas espécies mais sensíveis, existem riscos de efeitos adversos tais como inibição da reprodução e redução do recrutamento e do crescimento.

A maioria dos estudos sobre poluição por TBT descritos na literatura é dirigida aos ecossistemas costeiros e estuarinos, sendo escassa a informação relativa a áreas mais afastadas da linha de costa. Para colmatar esta lacuna de conhecimento, neste trabalho foi também realizado um levantamento dos níveis de *imposex* em *N. reticulatus* na plataforma continental adjacente a Aveiro, Lisboa, Setúbal e Faro. Pretendeu-se, assim, avaliar a evolução espacial da poluição por TBT numa área extensa e representativa da plataforma continental de Portugal. Verificou-se que muitas das estações de amostragem da plataforma adjacente a Aveiro, Lisboa e Setúbal apresentam valores de VDSI superiores a 0,3, classificando-se na classe C e, em alguns casos, na classe D, de acordo com os critérios da OSPAR. Estas observações indicaram que as populações destas áreas estiveram expostas a concentrações ambientais de TBT acima do EAC implementado. As análises químicas aos tecidos de *N. reticulatus* mostraram que o TBT esteve sempre acima do limite de detecção em todas as amostras analisadas. As concentrações de TBT variaram entre 12 e 356 ng TBT-Sn/g (peso seco) em Aveiro, entre 8,4 e 99 ng TBT-Sn/g (peso seco) em Lisboa e entre 3,6 e 120 ng TBT-Sn/g (peso seco) em Setúbal. Demonstrou-se que estes valores estavam significativamente correlacionados com os níveis de *imposex*, corroborando a relação causa-efeito já conhecida. Foi também observada uma correlação negativa entre as concentrações de TBT e a distância à embocadura dos estuários em cada uma destas áreas de estudo, comprovando-se que as zonas portuárias localizadas no interior dos estuários são a principal fonte de poluição por TBT na plataforma continental. O dibutilestanho (DBT) e o monobutilestanho (DBT) também foram detectados nos tecidos destes animais. Estes dois compostos são utilizados na indústria, essencialmente como estabilizadores de PVC e agentes catalíticos (Piver, 1973; Bennet, 1996), no entanto, a correlação significativa encontrada entre estes dois compostos e as concentrações de TBT sugere que o DBT e o MBT têm origem da degradação do TBT. O trifenilestanho (TPT) também foi detectado nos tecidos de *N. reticulatus,* embora em menor quantidade do que o TBT, enquanto os octilestanhos se apresentaram sempre abaixo do limite de detecção do método. Na região de Faro, que comparativamente a Aveiro, Lisboa ou Setúbal é uma zona com menor tráfego naval, apenas se registou um local (de entre dezasseis locais amostrados) em que o valor de VDSI *N. reticulatus* se situou acima de 0,3, o que denota um nível de poluição mais baixo do que nas restantes zonas do Norte e Centro do país.

Concluiu-se, portanto, que em 2006 os níveis de poluição por TBT, de uma forma geral, eram elevados e representavam um risco efectivo para os ecossistemas marinhos, não apenas ao longo da linha de costa e dentro dos estuários, onde estão localizadas as instalações navais mas também em zonas mais profundas da plataforma adjacente. Neste contexto, este estudo vem demonstrar que a poluição por TBT tem um vasto impacto no ecossistema marinho costeiro, porque os níveis de TBT extrapolados a partir dos dados de *imposex* são susceptíveis de causar diversos efeitos negativos nos mais variados grupos de organismos. A constatação de níveis elevados de poluição por TBT na costa portuguesa enfatiza a real necessidade de se implementarem medidas legislativas eficazes no combate a este problema.

8.2. Evolução temporal da poluição por TBT na costa portuguesa

Em 1993, Portugal adoptou a Directiva 89/677/EC que proibiu a utilização de tintas antivegetativas com TBT (e outros organoestanhos) em embarcações com menos de 25 m de comprimento, mas esta medida não foi eficaz na redução dos níveis da poluição por TBT (Barroso *et al.*, 2002b; Santos *et al.*, 2002). Em 2003 foi implementado o Regulamento EC/782/2003 na União Europeia, que proibiu a aplicação deste tipo de tintas em todas as embarcações. Neste trabalho pretendeu-se analisar a eficácia desta última medida, estudando-se a evolução dos níveis de *imposex* de *N. reticulatus*, ao longo dos últimos anos, como instrumento para avaliar se ocorreu recentemente alguma alteração da poluição por TBT na costa portuguesa. Os níveis de *imposex* obtidos neste trabalho em 2006 para a linha de costa foram comparados com os resultados obtidos em monitorizações realizadas na mesma área mas em períodos diferentes, nomeadamente em 2000 (Barroso *et al.*, 2002a) e em 2003 (Sousa *et al.*, 2005). Esta análise demonstrou que não existe diferença quando se comparam os níveis de *imposex* obtidos em 2000 e 2003 mas, pelo contrário, existe uma redução dos níveis de *imposex* quando se comparam os resultados de 2003 e 2006. O mesmo tipo de análise foi realizado para a plataforma continental adjacente a Aveiro, tendo-se verificado também uma redução dos níveis de *imposex* entre 2004 e 2006 e entre 2005 e 2006. Concluiu-se, assim, que houve uma diminuição da poluição por TBT durante este período de tempo na costa portuguesa. Dado que não houve alterações consideráveis no tráfego de embarcações durante aqueles anos, podemos considerar que esta diminuição é consequência da implementação do Regulamento EC/782/2003.

8.3 Evolução espacio-temporal da contaminação por TBT e por cobre nos sedimentos

Devido à implementação do Regulamento CE/782/2003, o cobre tem sido utilizado em maior escala como biocida nas tintas antivegetativas. Consequentemente, colocou-se a hipótese de poder haver um aumento nas concentrações de cobre nos sedimentos em consequência da recente legislação, acompanhado por uma tendência inversa no caso do TBT. Para testar esta hipótese fez-se um levantamento do grau de contaminação dos sedimentos por cobre e por TBT ao longo da costa em 2006 e compararam-se estes valores com os registados em amostras de sedimentos conservadas, obtidas para os mesmos locais em 2000. Os resultados mostraram que não existem diferenças significativas nos níveis de contaminação por cobre nos sedimentos entre aqueles dois anos. Inesperadamente, tendo em conta os resultados obtidos na análise temporal do *imposex* (que apontam para uma diminuição dos níveis de TBT no ambiente), também não se observou uma diferença significativa nas concentrações de TBT nos sedimentos entre 2000 e 2006. Este resultado mostrou que os sedimentos estão provavelmente a actuar como reservatório de TBT a longo prazo. Consequentemente, a remobilização deste composto, a partir dos sedimentos para a coluna de água, poderá abrandar a tendência de diminuição da poluição por TBT. Os resultados também demonstraram que os níveis de TBT e de cobre neste compartimento estavam significativamente correlacionados. Este facto pode sugerir que ambos têm uma fonte de contaminação comum (tintas antivegetativas), todavia esta relação poderá também dever-se apenas a uma coincidência geográfica de várias fontes de contaminação. Os níveis de cobre também foram analisados nos tecidos de *N. reticulatus* para determinar se este gastrópode poderia ser utilizado como bioindicador da contaminação por cobre. Os resultados demonstraram que não existia correlação significativa entre as concentrações de cobre nos tecidos de *N. reticulatus* e as concentrações daquele metal nos sedimentos. Concluiu-se então que, ao contrário do TBT, as concentrações de cobre nos tecidos de *N. reticulatus* não reflectiam as concentrações ambientais e, consequentemente, este gastrópode poderá não ser um indicador adequado da contaminação por este metal.

8.4 Influência do parasitismo por tremátodes na expressão do *imposex*

Apesar do *imposex* em *N. reticulatus* ter sido validado como biomarcador da poluição por TBT em estudos anteriores (Stroben *et al.*, 1992; Bryan *et al.*, 1993; Barreiro *et al.*, 2001; Barroso *et al.*, 2002a; Sousa *et al.*, 2005), deve avaliar-se exaustivamente se outros factores podem interferir na expressão do *imposex* nesta espécie. Existem alguns relatos, apesar de muito raros, da observação de *imposex* em gastrópodes antes de 1960, ou seja, antes do advento do TBT (Cardwell and Meador, 1989; Garaventa *et al.*, 2006). O *imposex* é um fenómeno induzido por disrupção endócrina nos gastrópodes (Porte *et al.*, 2006; Morley *et al.*, 2006) e esta disrupção pode, teoricamente, ser desencadeada por outros agentes para além das substâncias químicas (Jobling and Tyler, 2003). Consequentemente, colocou-se a hipótese do parasitismo por tremátodes poder influenciar a expressão do *imposex*, uma vez que os parasitas podem provocar alterações fisiológicas do hospedeiro para obter as condições necessárias ao seu metabolismo e sobrevivência (de Jong-Brink *et al.*, 2001). Esta hipótese tem levado à prática corrente de se eliminarem os gastrópodes parasitados quando estes são utilizados na monitorização da poluição por TBT, tendo esta regra sido também aplicada neste trabalho. No entanto, as fases iniciais de parasitismo podem não ser facilmente detectadas, por isso se considerou importante desenvolver este tema de investigação. De facto, o parasitismo por tremátodes ocorre com frequência nos gastrópodes mas raramente é considerado nos estudos de disrupção endócrina, e nunca este aspecto foi estudado em *N. reticulatus*. Verificou-se que na costa portuguesa existem, pelo menos, seis espécies de tremátodes que utilizam o gastrópode *N. reticulatus* como hospedeiro intermediário, nomeadamente, *Cardiocephalus longicollis*, *Cercaria sevillana* – a cercária de *Gynaetotyla longiintestinata*, *Diphtherostomum brusinae*, *Himasthla quissetensis*, *Lepocreadium album* e uma cercária desconhecida, pertencente à família Zoogonidae. A observação histológica de gastrópodes parasitados mostrou a presença dos estádios larvares dos tremátodes no complexo glândula digestiva/gónada. Também mostrou que todo o tecido reprodutivo é substituído pelos estádios assexuados dos parasitas, revelando que estes tremátodes têm um efeito castrante em *N. reticulatus*, ou seja, a sua presença inibe a gametogénese devido à alteração morfológica do tecido reprodutivo. Assim sendo, esta castração pode, hipoteticamente, conduzir a uma desregulação endócrina no gastrópode que, por sua vez, poderá influenciar a expressão do *imposex*. No entanto, com base em dados de campo, verificou-se que não havia diferenças entre os níveis de *imposex* de fêmeas parasitadas e não parasitadas, concluindo-se que o parasitismo não tem influência na expressão do *imposex*. Estes resultados validam, mais uma vez, a utilização do *imposex* como biomarcador em estudos de monitorização da poluição por TBT. Contudo, nos machos, o parasitismo teve um efeito redutor no tamanho do pénis o que reforça a necessidade de continuar a prática corrente de não se utilizarem animais parasitados em estudos de *imposex*, principalmente quando se pretende determinar índices que comparam o tamanho dos pénis das fêmeas com o dos machos.

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ANEXOS

ANNEXES

Índice das Figuras – Figures Content

Índice das Tabelas – Tables Content